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„ Heterosis in the Freezing tolerance of *Arabidopsis thaliana* (L.) Heynh."

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to my family

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IV Abbreviations

ABA	Abcisic acid
ACC	Acclimated
AFP	Anti- freeze proteins
APPI	Atmospheric pressure photoionization
BPH	Best-parent-Heterosis
Can	*A.thaliana* accession Canary Island
CAS	Cold acclimation specific
CBF	CRT binding factor
CDPK	Calcium-dependent protein kinase
CI	Chemical ionization
Co-2	*A.thaliana* accession Coimbra
Col-0	*A.thaliana* accession Columbia
COR	Cold regulated
Cvi	*A.thaliana* accession Cape Verde Island
Dehydrin	Dehydration induced
DNA	Desoxyribonucleic acid
DREB	Dehydration responsive element binding
EI	Electron Impact
ESI	Electron-Spray Ionization
F1, F2	Filial generation (first, second)
GC	Gas chromatography
GD	Genetic distance
HPLC	High performance liquid chromatography
hQTL	heterotic quantitative trait locus
ICA	independent component analysis
ICE	Inducer of CBF expression
IL	Effect- increased loci
IUPAC	International Union of Pure and Applied Chemistry
KEGG	Kyoto Encyclopedia of genes and genomes
LC	Liquid chromatography
LEA	Late embryogenesis abundant
L*er*	*A.thaliana* accession Landsberg *erecta*

LIFDI	Liquid injection field desorption ionization
LT_{50}	Lethal temperature
LTI	Low temperature induced
MALDI	Matrix- assisted laser desorption/ ionization
MAPK	Mitogen activated protein kinase
Mb	Mega base pairs
METB	Metabolite
MPH	Mid- parent-heterosis
MS	Mass spectrometry
MST	Mass spectral tags
NA	Nonacclimated
NIST	National Institute of Standards and Technology
OD	Overdominance
OE	Overexpression
P	Parental generation
PAP	Production of anthocyanin pigment
QUAD	Quadrupole detector
RD	Responsive-to-dehydration
ROS	Reactive oxygen species
Rsch	*A.thaliana* accession Rschew
SA	Salicylic acid
SAR	Systemic acquired resistance
sfr	Sensitive to freezing
SNP	Single nucleotide polymorphism
TCA- cycle	Tricarboxylic acid- cycle
Te	*A.thaliana* accession Tenela
t_f	Flow time
TOF	Time-of-flight
t_R	Retention time
TRAP	Ion trap
US	United States
UV-light	Ultraviolett light

1. Summary

Arabidopsis thaliana is one of the most used model plants worldwide. It has a small genome (125 Mb on 5 chromosomes), low requirements on growth conditions, a short generation time and is widely spread over the whole northern hemisphere. In addition *Arabidopsis* is selfing and thereby a very good object for crossing experiments.

The term heterosis describes the enhancement of F1-hybrids in comparison to the parents. Mid – Parent - Heterosis (MPH), characterize the melioration to the parental mean and Best – Parent - Heterosis (BPH), is a progression above the better parent. This affects e.g. enhanced biomass, but also tolerance against several kinds of stress. The advancement declines in the following generations.

Despite decades of use of this principle in agriculture, the genetic basis of heterosis remains still unclear. Different studies suggest dominance, overdominance or epistasis, separate or in combination.

In the present study heterosis in freezing tolerance was investigated by comparing *Arabidopsis*- crosses. The parental accessions originated from very distinct climatic regions. Their freezing tolerance (Lethal temperature, LT_{50}) was determined in previous studies and a clear correlation was found with the original habitat temperature. From six accessions (Canary Island, *Can*; Cape Verde Island, *Cvi*; Coimbra, *Co-2*; Landsberg *erecta*, *Ler*; Rschew, *Rsch* und Tenela, *Te*) and the laboratory lines C24 and Columbia (*Col-0*) 24 reciprocal crosses were performed to define freezing tolerance, proline and sugar contents (glucose, fructose, sucrose and raffinose). These metabolites demonstrably accumulate under cold in plants. All measurements were performed on non acclimated (NA) and cold acclimated (ACC) plants. Cold acclimation was achieved by transferring the plants to a 4°C cold phytotrone for two weeks. Based on this data, type and strength of heterosis were calculated and used to select eight crosses for further experiments (*C24 x Col, Col x C24, C24 x Co-2, C24 x Ler, C24 x Te, Col x Co-2, Col x Ler, Col x Te*). On these crosses and the corresponding parental lines metabolite profiling (gas chromatography- mass spectrometry, GC-MS) was performed and the appearance and content of several flavonoids (by liquid chromatography- mass-spectrometry, LC-MS) detected.

The measurements showed significant more frequent and stronger heterosis in *C24*- than in *Col*- crosses. This pertains the obtained freezing tolerance as well as the accumulation of

proline and the four sugars. Heterosis values in acclimated plants exceeded the values of non acclimated plants by a multiple. The same was found for flavonoid contents.

Correlation analyses showed clear correlation between freezing tolerance and the contents of carbohydrates, proline and several flavonols. The strength of heterosis in freezing tolerance an in metabolite content also correlated significantly.

The global metabolite profiling by GC-MS confirmed the accumulation described above and the calculated correlation for all four sugars and proline. In three independent experiments 40 metabolites were consistently found and further investigated. The strongest accumulation during cold acclimation was obtained in C24 and *Coimbra* (Portugal), the lowest in *Tenela* (Finland). Many substances correlated significantly with freezing tolerance and/or with MPH in metabolite content and in LT_{50}.

Interestingly six out of 11 substances which correlated significantly were important compounds of the tricarboxylic citrate (TCA) cycle. A change in the amount of several parts of this metabolic pathway was observed before in other organisms (animals and plants) and in combination with other stresses, but not connected with heterosis. The results presented here display negative correlations between MPH in freezing tolerance and metabolite accumulation. This points to a consolidated role of parts of the TCA- cycle in crosses compared to the parents and thereby to a change of flux rates in the cycle.

To proceed the inquiry a transcript profiling should be performed, comparing gene expression of accessions and crosses before and after cold acclimation. A similar study by Hannah *et al.* (2005) compared this trait in a range of natural homozygous lines.

Furthermore an analysis of quantitative trait loci (QTLs) could shed light on the metabolic and genetic connection of TCA-cycle and detected heterosis.

2. General introduction

2.1. *Arabidopsis thaliana (L.)* Heynh.

Arabidopsis thaliana (thale cress) was described first by Johannes Thal (1588) in the 16th century and later introduced by Laiber and colleagues into science as a model organism. Besides others like rice (*Oryza sativa L.*), maize (*Zea mays*), tobacco (*Nicotiana tabacum*,) tomato (*Solanum tuberosum*) or wheat (*Triticum L.*), it is still one of the most investigated plants.

Arabidopsis belongs to the mustard-family (*Brassicaceae)* and is a close relative to important crops like rapeseed (*Brassica napus*) and cabbage (*Brassica oleraceae*). Originating from Eurasia and North Africa, it was distributed by man over the whole northern hemisphere. Seeds from over 400 natural accessions are publicly available through seed banks. The accessions vary in the latitude, longitude and altitude of their original habitats (Dekker & Altmann; 2001, Li *et al.*, 1998) and accordingly also in a wide set of traits. This affects developmental, physiological and biochemical characteristics like flowering time and biomass, seed dormancy, water use efficiency or growth rate (longevity). The accessions differ in gene expression, enzyme activity and metabolite accumulation as well as in the susceptibility to several kinds of abiotic (Hannah *et al.*, 2006) and biotic stress.

Genotyping of 351 accessions using 115 Single nucleotide polymorphism (SNP) markers revealed clear genetic differences associated with the varying geographic origins (Schmid *et al.*, 2005). This leads back to the fast colonization of Central Europe following the ice age. Also selfing was evolved during this period (Shimizu and Purugganan, 2005). Based on sequence data three clusters where defined with accessions from Central Europe, Central Asia and the Iberian Peninsula including the Mediterranean region.

Arabidopsis is a weedy annual and a diploid, self-pollinating plant with a short life cycle (~3 months). It overwinters either as seeds and germinates and flowers in spring (summer annual) or as a vegetative rosette (winter annual) after germination in fall. In both cases, it survives the warm and dry summer months in the seed stage. The plants are small and generate a large offspring with up to 4000 seeds per plant. This enables large experiments on small areas during a short time span.

Thale cress is well suited for detailed genetic studies since the genome is relatively small with 5 chromosomes and a total size of 125 Mb, of which only a small part consists of repetitive DNA. It was fully sequenced by The *Arabidopsis* Genome Intitiative in 2000

(Initiative T.A.G., 2000). In addition, large mutant collections are publicly available to study the function of particular genes and gene products.

Using these advantages, many phenotypic and molecular differences were described during the last years between natural accessions (Mitchell-Olds and Pedersen, 1998; Koorneef *et al*., 2004), but also in mutants and transgenic plants.

The knowledge gained through *Arabidopsis* helped to accelerate the sequencing of rice (*Oryza sativa L.*), another important model and crop plant (Project IRGS, 2005). From both species nuclear, chloroplast and mitochondrial genome sequences are available (Rensink and Buell, 2004).

2.2. Heterosis

The term heterosis (hybrid vigor) was introduced into the literature by Shull in 1914 (Shull, 1914). It describes the better physiological performance of a hybrid (F1) compared to both parents, but the effect is not fixable by inbreeding.

Heterosis is especially important in crop plant breeding. It has led to large increases in economically important traits like biomass accumulation, seed yield, resistances or nutrient uptake (Narang and Altmann, 2001). This increases e.g. the possible geographical/ climatic distribution of the cultivation area if the cross is able to cope with environmental stresses, such as extreme temperatures.

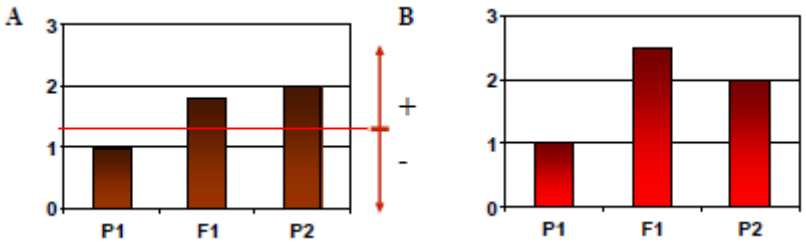

Figure 1. Kinds of heterosis
Both kinds of heterosis are illustrated, with the hybrid (F1) compared to both parental accessions (P1, P2). Mid-parent heterosis (MPH, **(A)**) occurs, if the performance of the hybrid is significantly different from the mean of both parents. It can be positive (+) or begative (-). Best-parent heterosis (BPH, **(B)**) occurs when the cross excels both parents.

Two kinds of heterosis (Fig. 1 A, B) are generally distinguished. Best-parent heterosis (BPH) occurs if the hybrids perform better then the better parent. In Mid-parent heterosis

(MPH) the crosses shows better (positive MPH) or worse (negative MPH) performance than the mean value of both parents.

MPH and BPH can be calculated in two ways, depending on the interest of the researcher, as absolute or relative hybrid vigor (Tab.1).

Relative heterosis indicates the percentage of melioration or impairment of the hybrid, while absolute heterosis reflects the absolute change compared to the parental lines. The latter is probably more significant for plant breeders, since it represents the additional amount of e.g. expectable yield and thereby economic profit. This absolute heterosis was also used throughout my study.

Table 1. Calculation of absolute and relative heterosis, used to describe the level of improvement of hybrids (F1) in comparison to both parental lines (P1, P2).

	Best-parent Heterosis	**Mid-parent Heterosis**
Absolute	F1 – max(P1; P2)	F1 – (P1+P2)/ 2
Relative	$\frac{F1-max(P1; P2)}{max(P1; P2)}$	$\frac{F1 – (P1+P2)/2}{(P1+P2)/2}$

One of the main practical problems with heterosis is the decrease of its effect with increasing homozygosity during further inbreeding (F2, F3...). This leads first to a loss of the superior trait and later to inbreeding depression. In consequence, the breeder is forced to collect the parental material and repeat the cross whenever new seeds are needed.

Although the principle is used since decades, the genetical basis remains unclear. There are three main hypotheses – dominance, overdominance (OD) and epistasis. All of them have already been "proven" as the basis of heterosis, in combination with one of the others or alone, but all also have been contradicted. A special stimulus of heterozygosity on cell division, growth and other physiological processes in the organism is assumed. Many biologists declare this process as absolutely necessary in evolution since it leads to adaptive selection (Tsaftaris, 1995). Probably an optimal genetical distance of the parental lines exists which determines the heterotic effect. Outside these genetical limits the chance of an effective adaptation of the parental genomes decreases (Barth *et al.*, 2003). In a large-scale experiment on grain yield of rice subspecies *indica* hybrids, Renming *et al.* (2008) found a strong correlation between parental genetic distance (GD) based on effect-increasing loci (IL) and F1 performance.

The dominance hypothesis attempts to explain heterosis as a result of complementation when newly combined allels of a gene or several genes positively influence a trait (Birchler *et al.*, 2003). An improvement of a trait would be possible if one allel is defective or at least inferior. Heterozygosity of the hybrid could complement a recessive unfavorable mutation from one parent by the second dominant allel of the other parent. It is assumed that an internal control mechanism exists defining a maximum for each trait. In homozygous parental accessions, the two identical allels maximize the strength of the control loci and prevent changes. In the hybrid two different allels are combined. This reduces the effect and enables the F1, e.g. to exhibit higher biomass or length growth (Millborrow, 1998). Not one single allel, but the heterozygotic mix leads to an improvement. Thereby heterosis could emerge even with one allel partly defective.

Critics of this hypothesis have pointed out that dominance as the only molecular basis of hybrid vigor would imply the chance to create inbred lines expressing all superior genes. Protagonists respond that with the large number of potentially involved genes and possible combinations, transferring all positive traits into one plant would be unlikely (Birchler *et al.*, 2003). This, however, does not explain the consistent improvement of properties in inbred lines during the last centuries. Selection by breeders continuously enhanced plant performance in inbred lines without decreasing the effects of heterosis in crosses.

In epistasis the phenotypic manifestation of one gene is covered by another from another gene pair (Pschyrembel, 2007). Li *et al.* (2001) assume that for complex traits this effect is even stronger in self-pollinating plants than in cross pollinating. Narang and Altmann (2001) explained heterotic effects in *Arabidopsis* biomass with a combination of epistasis and dominance.

The overdominance hypothesis includes allelic interactions emerging through crossing in hybrids into the explanation of heterosis. Only combinations of different allels produce regulatory gene-allel-interactions. Either many less effective loci or a few loci with strong effects could produce hybrid vigor. But these single loci are outperforming in the cross compared to the homozygotes (Birchler *et al.*, 2003). In contrast pseudo-overdominance assumes a network of linked loci.

Lu *et al.* (2004) investigated heterosis in maize (*Zea mays* L.) by analysis of heterotic quantitative trait loci (hQTLs). They found overdominant hQTLs for grain yield as well as partially to completely dominant hQTLs for plant height and other traits. In addition, they

detected only little epistasis for all traits. Another hQTL mapping study in maize (Yan *et al.*, 2006) showed strong overdominance effects for traits with higher heterosis and a possible key role of digenic effects at the two-locus level also referred to as epistasis.

In conclusion all three hypotheses were already proven in some experiments and contradicted in others. It seems likely that no single explanation is valid for all traits in all plants, but that the mechanism is different for different genotypes, traits and conditions.

2.3. Stress in plants

All organisms – animals and plants – suffer from stress. Stress means the presence of suboptimal conditions which force the organism to apply more energy and change its behavior to adapt and to survive. There are abiotic and biotic stressors (Tab. 2), which can occur separately, in combination or in direct succession (Mittler, 2006).

Table 2. List of different abiotic and biotic plant stressors

Abiotic stresses	**Biotic stresses**
Heavy metals	Bacteria
Nutrition (lack or surplus)	Fungi
Heat	Herbivores
Cold/freezing	Insects
Ozone	Viruses
High light/ low light	
Drought	
Flooding	
High salt concentration	

One stressor can weaken the plant and make it more susceptible for another one. For example the sensitivity to biotic stressors like fungi increases in trees with bark damaged by frost. Biotic and abiotic stresses can lead to the formation of free radicals that cause oxidative stress, which damages lipids, proteins and nucleic acids (Mittler, 2002).

Plant internal defense reaction (genetically and metabolic) can be similar to several stresses, as several stressors may lead to similar problems (Houde *et al.*, 2006). High salt concentration, extreme temperatures and desiccation result in intracellular dehydration. Salt tolerant accessions of rice were found to exhibit also a higher chilling tolerance compared to

salt sensitive plants (Kazemitabar *et al.*, 2003), which indicates shared responses to various stresses in plants.

In contrast to most animals, plants are unable to avoid stress by changing the location. They are restricted to react with changes in metabolism, by reduction of growth, production of new metabolites, rearrangement or loss of organs. Dependent on the type and duration of stress and the general condition (developmental state, nutrient supply), a plant can withstand the stress better or worse. Tobacco (*Nicotiana tabacum*) and cucumber (*Cucumis sativus*) accumulate salicylic acid (SA) under biotic and UV- stress and high ozone as part of a signaling process to initiate systemic acquired resistance (SAR). Many plants accumulate high amounts of phenylpropanoids against stress; anthocyanines (high visible light, cold, nutritional stress), flavonols (wounding) or isoflavonoids (UV) (Dixon and Palva, 1995).

Calcium plays an important role in signal transduction to excite physiological processes in reaction to hormones, pathogenes and several abiotic stresses (Song *et al.*, 2008). Many plants are able to adapt to stresses up to a certain level. This process is termed acquired tolerance enhancement or acclimation (Guy, 1999; Thomashow, 1999). By exposing the plants for some days to mild temperature stress the plants increase their tolerance against stronger heat and cold (Kaplan *et al.*, 2004). This adaptation goes along with a number of changes in membrane structure and function, gene expression, lipid and metabolite composition (Gilmour *et al.*, 2000; Shinozaki and Dennis, 2003).

The ability of crops to cope with stress is a very important trait for farmers and breeders. It determines the necessary expenditure of time and money from sowing till harvest, including e.g. the amount of fertilizer and herbicides/ insecticides. The kind and level of stress depend on the crops chosen for a distinct area. Therefore it is important to select genotypes, which are best adapted to the local conditions like soil, water supply, light intensity and temperature. This increases the strength of defense since it enables a good nutritional status of the plant. Differences are not only found between species, but also between accessions or breeding lines of the same species. For example, Kover and Schaal (2002) found significant differences in disease resistance among 19 *Arabidopsis* accessions concerning strength of symptoms, size of the bacterial population and fitness of the host.

Attempts to improve stress tolerance have been made in many plants. This includes classical breeding and metabolic engineering, genetic attempts (Kasuga *et al.*, 1999) to knock out single genes or intensify gene function to manipulate whole signal transduction pathways.

2.3.1. Cold stress

2.3.1.1. The impact of low temperatures/ cold stress on plants

Farmers worldwide suffer huge yield losses. Steponkus *et al.* (1993) estimated around 14 billion US-dollars deficit yearly only by freezing damage, despite several prevention methods. This includes energy intensive procedures to improve the microclimate in winter by wind machines, artificial rain and helicopters. A small enhancement of freezing tolerance in several crops would already lead to a significant increase of the agricultural productivity and profitability (Steponkus *et al.*, 1993).

Cold and freezing stress of plants play a crucial role in temperate regions, areas with high altitude, in subarctic habitats or areas with drastic night- and- day temperature changes. It causes physiological and biochemical changes (Tomashow, 2001; Palva *et al*., 2002). Low temperatures affect, amongst others, growth, water balance, the accumulation of metabolites, changes in the expression of genes and proteins. After a short initial phase during the cold response, also the circadian clock is altered and the amplitude of diurnal cycles reduced (Bieniawska *et al.*, 2008).

An unexpected freezing period can harm plants seriously. The range of damages includes the wilting and loss of leaves from winter green plants, bark damage by sunburn in cold winters with high radiation, the loss of buds in the following spring or the death of the whole plant. Wilting results from a proceeding of photosynthesis and respiration in the plants accompanied by a decreased or stopped water uptake by the roots in the frozen soil. (Johnson *et al.*, 1988). Secondary injuries are sustained when mechanical damages are combined with cold and humidity. In winter plant growth is reduced, wounds cannot be repaired by the plant and represent an entrance for biotic stressors like fungi. At subzero temperatures plants have to cope with various problems. Uptake of water or nutrients via the roots is limited or impossible on frozen soil, but photosynthesis continues, especially in evergreen plants (Oliveira and Penuelas, 2004). While light quantity remains, all biochemical reactions are reduced. Through dehydration stomata close, CO_2 diffusion is affected and photosynthesis altered. This results in the production of reactive oxygen species (ROS), toxic for the plant. Particularly in photosystem II (PSII) changes are reported (Öquist and Huner, 2003), but also photosystem I seems to be affected under stress (Scheibe *et al.*, 2005). Hannah *et al.* (2005) found a significant down-regulation of transcripts responsible for photosynthesis and lipid metabolism, while transcripts for primary and secondary metabolism were up-regulated.

At freezing temperatures ice crystallizes in the apoplast. Extra-cellular ice formation is caused by a higher solute content inside the cells then outside and by the presence of nucleators. It is not necessarily lethal but induces mechanical and chemical stress. The developing water gradient leads to an intracellular dehydration and a decrease of volume since the water is drawn out the cell. Dehydration can lead to a separation of phospho- and glycolipids into more and less hydrated domains. This leads to the formation of a Hexagonal II - phase in the cell membranes and to a loss of cellular compartimentation. After thawing, semi-permeability of cell membranes is changed which results in cell death. Cold acclimation prevents or delays these processes by protein synthesis (Sror *et al.*, 2003), additional accumulation of sugars and other compounds protective to the membranes and liposomes (Hincha and Hagemann, 2004).

Instead of developing freezing tolerance, some plants are able to avoid freezing and ice formation. It was shown that some trees "supercool". Ice crystallizes only in the extra-cellular parts of outer layers of the bark, while other tissues, like the xylem ray parenchyma cells stay unfrozen. These cell types do not contain intrinsic nucleators and this enables trees to overwinter at temperatures down to -40°C, a process referred to as "freeze avoidance".

During cold acclimation the membrane lipid composition changes (Uemura *et al*., 1995), compatible osmolytes are accumulated and the level of antioxidants increase while plant growth is stopped (Browse & Xin, 2001). Anti-freeze proteins (AFPs) can be accumulated to prevent injury by ice formation (Houde et al, 2006). In insects, fishes and plants anti-freeze proteins were detected which support the survival of organisms by inhibition of ice re-crystallization, the growth of ice crystals inside the extra-cellular spaces (Tomczak *et al.*, 2002).

Chilling tolerance is defined as the ability to tolerate temperatures between 15°C and 0°C, freezing tolerance describes the reactions of plants at temperatures below 0°C after the crystallization of ice in the tissues. Plants vary a lot in their ability to survive low temperatures (Ouellet, 2007). The ability to tolerate freezing or chilling temperatures is strongly linked to the climate of the original habitat of a plant species/ accession/ variety. Plants from tropical regions suffer temperature stress already far above 0°C.

Arabidopsis thaliana provides an example of how freezing tolerance differs between natural accessions of one species. Hannah *et al.* (2006) compared nine accessions from various origins and found significant differences in freezing tolerance and cold acclimation ability. The ability to cold acclimate is a clear advantage for many plants originating from

temperate and cold regions. After transfer to low positive temperatures for a short time period (some days or weeks), these plants develop an improved tolerance to temperatures below freezing point. An increase of several degrees in freezing tolerance is possible (Thomashow, 1999; Rhode *et al.*, 2004; Hannah *et al.*, 2005). This process is needed for winter survival (Houde *et al.*, 2006), but it is reversible (Guy, 1990) and not all plants are able to undergo this process. Especially plants from geographic regions without seasonal changes in temperature are often not adaptable to lower temperatures. This includes tomato (*Solanum lycopersicum* L.) and pepper (*Capsicum anuum*), but also mediterranean trees like *Citrus*. Often an increase of tolerance is possible by grafting on a tolerant partner, but the possible combinations and the natural maximum of freezing susceptibility are limited.

Metabolic and genetic analysis indicated a high variability between accessions grown under the same conditions (Hannah *et al.* 2006). Similar results were obtained for other traits in *Arabidopsis* as well as the chilling/ freezing tolerance of other species. The determination of chilling tolerance in rice showed higher survival rates of *indica* -, than of *japonica*-subspecies (Kazemitabar *et al.*, 2003).

Frost tolerance is a multigenic trait and cold acclimation involves changes in membrane composition (Steponkus, 1993), global gene expression (Hannah *et al.*, 2005), protein (Bae *et al.*, 2003) and metabolite content (Kaplan *et al.*, 2004; Guy *et al.*, 2008). Genes encoding proteins involved in the synthesis of amino acid, lipids and secondary metabolites show altered expression (Houde *et al.*, 2006). To react, plants have to perceive the temperature change and to transduce the signals to adjust the expression of cold-responsive genes (Smallwood and Bowles, 2002).

Plants respond to cold stress in at least two ways – abscisic acid (ABA) - dependent and ABA-independent. The ABA-dependent pathways include Calcium (Ca^{+}) - sensing. Ca^{+} - influx plays an important role in signal transduction and adaptation to several stresses (Song *et al.*, 2008). It acts as a secondary messenger in many plants and its concentration increases rapidly after a shift to low temperatures and leads to the expression of many cold regulated (COR) genes, like COR6.6, COR78, KIN1. Calcium–chelators or Ca^{2+} -channel blockers prevent influx of extracellular calcium and thereby cold acclimation (Monroy and Dhindsa, 1995). Ca^{+} mediates the stress response through transcription factors (TFs) binding to phosphatases, calcium-dependent protein kinases (CDPKs) and abscicic acid regulating elements (ABREs) in gene promoters (Guy *et al.*, 2008).

In *Arabidopsis* around 40 CDPKs were found to be up-regulated at 4°C. They are necessary for cold acclimation in plants by controlling the expression of stress-responsive

genes. It was shown that exogenous ABA enhances freezing tolerance and has an impact in long-term responses to low temperatures. Mutants (*aba deficient* (*aba1), aba insensitive 1(abi1*)) either deficient in ABA-synthesis or insensitive to ABA decreased in cold tolerance. Leung *et al*. (2006) showed that the mitogen-activated kinase MAPK6 is a potential target of ABI1 and transmits different stress signals. Mitogen activated protein kinases (MAPKs) are known to be up-regulated under various stresses.

The ABA-independent gene expression is induced by activation of dehydration responsive element binding (DREB) proteins binding to DRE motifs in the promoter region of cold resonsive genes. DREB (DRE binding factor)/ CBFs (CRT binding factors) are transcription factors activating the expression of cold regulated (COR) genes and functioning as key regulator components in cold acclimation. CBFs bind to a cis – acting element in the promoter region of COR genes. One of them (CBF3) is induced by ICE (inducer of CBF expression), which is a constitutively expressed transcription factor that is activated at low temperatures. The expression levels of the CBF genes increase already 15 minutes after transfer to cold conditions. Approximately 500 genes were found to be strongly induced (50-100-fold after cold acclimation) in *Arabidopsis* (Browse & Xin, 2001; Hannah *et al.*, 2005). The accumulation of COR transcripts starts after that of the CBF genes and they remain elevated during cold (Christie *et al.*, 1994).

DREBs/ CBFs are divided into two groups, DREB1 and DREB2. DREB1A (CBF3), DREB1B (CBF1) and DREB1C (CBF2) are induced by low temperatures, while members of the DREB2-group are involved in enhancement of plant desiccation tolerance. CBF1, CBF2 and CBF3 are physically linked in direct repeat on chromosome 4. CBF2 negatively regulates the expression of CBF1 and CBF3, but is positively correlated with an increase in freezing tolerance. Overexpressors (OE) of CBF1 and 3 showed an increased freezing tolerance. OEs of CBF3 exhibit a strongly increased freezing tolerance, an enhanced expression of COR-genes and elevated levels of several metabolites (e.g. galactose, raffinose, fructose, glucose, proline and mannose). Under normal conditions these mutants show reduced growth. Xin and Browse (2001) showed that the eskimo1 mutation (esk1) results in an enhanced freezing tolerance, soluble sugar and proline levels, without influencing COR gene expression. ZAT12, another TF associated with elevated COR-gene expression overlaps with the CBF2-regulon and negatively regulates CBF-expression. In many plant species in addition to *Arabidopsis* cold regulated (COR) genes named also LTI (low temperature induced), CAS (cold acclimation specific) or RD (responsive–to–dehydration) have been described. Overexpression leads to increased freezing or dehydration tolerance.

COR proteins are often highly hydrophilic and/or have a high glycine content. Some COR proteins are members of the dehydrin (Dehydration induced)/LEA (late embryogenesis abundant) protein family - a group of proteins that are thought to play a role in plant stress tolerance and seed desiccation tolerance (Hundertmark and Hincha, 2008).

A number of other genes and proteins has been described that are involved in low temperature and freezing tolerance. Sensitive to freezing (*sfr*) mutants show decreased freezing tolerance compared to wild type and are unable to cold acclimate. The *Arabidopsis* HOS1 gene (ABA-independent) negatively regulates transcription of CBF and has been the first gene with proven influence on both cold acclimation and vernalization. The HOS1 protein interacts with ICE1 (Dong *et al.*, 2006). *hos1* and *hos2* are hypersensitive to low temperatures and *hos1-1* results in a superinduction of COR genes (Ishitani *et al.*, 1998).

An improvement of tolerance to low temperatures has been attempted in several ways; by overexpression of genes known to be involved in cold acclimation or by manipulation of whole signal transduction pathways to activate stress-responsive genes through the overexpression of genes encoding transcription factors. Metabolic engineering could increase the synthesis of metabolites related to improved frost resistance, like soluble carbohydrates, proline, flavonoids, abscisic acid. Another possibility is the use of conventional breeding, which includes the use of heterosis. Reciprocal F1-crosses of *Arabidopsis thaliana* accessions C24 and Columbia showed a significant increase in nonacclimated and acclimated freezing tolerance and in their content of soluble sugars. No indication was found for the involvement of CBF1, CBF2 and several COR- genes in the heterotic effect (Rohde *et al.*, 2004).

2.3.1.2. Metabolic effects of cold in plants

Low temperatures result in significant changes in many metabolite networks in plants. Cells have to react on deviations of all conditions from the preferred optimum. The plant has to react on internal changes to maintain all processes and if possible to develop an enhanced tolerance to decrease the energy input. With increasing freezing tolerance photosynthesis and hormonal reactions are down-regulated (Hannah *et al.*, 2006), while flavonoid metabolism, synthesis of antioxidants and accumulation of compatible solutes are induced. Four groups of metabolites were found to play crucial roles in the preservation of cellular functions and protection against freezing temperatures.

Osmolytes and **compatible solutes** (e.g. sugars, some amino acids) reduce cellular dehydration by changing the cellular osmotic potential and by stabilizing membranes (Hincha

et al., 2002) and enzymes. **Antioxidants** protect against oxidative damage introduced by reactive oxygen species (ROS). In addition **energy sources** (starch) and **membrane lipids** (Guy *et al.*, 2008) are altered by plant responses to cold.

The sugars glucose, fructose, sucrose and raffinose as well as the amino acid proline are important cryoprotectants with a strong response to cold (Guy *et al.*, 2008) and all sugars correlated positively with freezing tolerance of *Arabidopsis* accessions and F1 hybrids (Hannah *et al.*, 2006, Korn *et al.*, 2008).

An accumulation of polyamines and the antioxidants (e.g. ascorbate, glutathione and glycine betaine) has been reported in grasses (Guy, 1990), barley (*Hordeum vulgare*), radish (*Raphanus sativus*) (Chu *et al.*, 1974) and wheat (*Triticum spp*.) (Houde *et al.*, 2006).

The levels of other amino acids (arginine, cysteine, lysine, methionine, serine, phenylalanine and tryptophane) were found to be 5-fold increased in wheat under cold and drought. The increase of aspartate, ornithine and citruline in *Arabidopsis* could indicate an up-regulation of the urea cycle. An up-regulation of the tricarboxylic acid (TCA) -cycle at low temperatures can be concluded from the accumulation of α-ketoglutarate, fumarate, malate and citrate.

Secondary metabolites like water soluble phenolics (*Rhododendron L.,* Solecka *et al.*, 1999) and anthocyanins increase under cold and other stresses (Dixon and Palva, 1995, Korn *et al.*, 2008). The amount of some flavonoids in *Arabidopsis* was found to be correlated with freezing tolerance in several accessions and F1 hybrids (Korn *et al.*, 2008). This agrees with the finding of significant up-regulation of genes of the flavonoid biosynthesis pathway by Hannah *et al.* (2006).

The response of metabolites to suboptimal conditions does not always start simultaneously with the sensing of stress. It is evenly distributed over three time groups – early (1-4 h), intermediate (12-24 h) and late (48-96 h) (Kaplan *et al.*, 2004). Carbohydrates (e.g. galactose, maltose, xylose, fructose-6-phosphate, glucose-6-phosphate) are mainly increasing during the early phase of stress, while sugar alcohols (sorbitol and galactinol) are significantly raised 96 hours after cold induction (Guy *et al.*, 2008). Proline belongs to the intermediate group. The beginning of its accumulation is shifted, but the level stays elevated several hours after the return of the plants to normal conditions.

2.4. Metabolite Profiling

2.4.1. Methods for metabolite determination

Several methods for quantitative and qualitative metabolite determination were invented and improved during the last decades. Sugars, alcohols, amino- and other acids as well secondary metabolites and lipids can be measured. Depending on the interests and the present compound mix, the choice is given between gas chromatography (GC) or liquid chromatography (LC).

Both can be used for targeted or untargeted analysis of samples. Targeted analysis searches for a special metabolite or group of metabolites in a sample. An analyte can be a chemical compound or a compound mixture, liquid, gaseous or solid. For detection a standard of the compound of interest is measured in parallel. Afterwards the chromatograms of the sample will be compared to that of the standard.

For untargeted analysis the peaks of resulting chromatograms are compared to and identified by using an analyte library. This library contains known (METBs) and unknown (MSTs), but cataloged compounds (Kopka *et al*., 2005).

The National Institute of Standards and Technology (NIST), situated in the United States, releases the selection for substance identification by mass spectra, the NIST Mass Spectral Library (www.nist.gov). Besides this there are a number of organizations and data bases publishing identifiers for metabolites/analytes, nomenclature, terms and standardized measuring methods. Information from the "International Union of Pure and Applied Chemistry" (IUPAC) and the "Kyoto Encyclopedia of Genes and Genomes" (KEGG) is freely available (www.genome.jp/kegg), while the data base of the "Chemical Abstract Service" (CAS), which includes the most reliable names of more than 30 million substances, is a commercial service (www.cas.org).

Chromatography

"Chromatography" depicts the separation of a substance mixture by distribution into its single compounds between a stationary and a mobile phase. The mobile phase contains the sample in a carrier substance, while the stationary phase occupies a column the mixture has to pass. Different components in a mixture are separated by their physical properties, such as charge, size or hydrophilicity. They are identified by comparing retention time (t_R), the time a pure molecule would need to pass the stationary phase, to flow time (t_F), the duration of the mobile phase. The principle was described first by the Russian botanist Michail, Tswett in 1901. The term was introduced in 1906.

A) Gas chromatography (GC)

In GC the mobile phase consists of an inert gas, usually helium, nitrogen or hydrogen. After injection, the sample is volatilized by heating, added to the mobile phase and passed through a column. This column generally consists of silica glass and is coated with the stationary phase, in my case polysiloxanes. The method is based on the different boiling points of the single substances in the sample and the interaction with the stationary phase according to the polarity. The latter leads to a retardation of some compounds and allows thereby to discriminate according to the known t_R. GC is a fast method to distinguish exactly between hundreds of mass spectral components – known (METB) and unknown (MST) with known t_R and peaks distribution (Erban *et al.*, 2007).

B) Liquid chromatography (LC)

LC-MS combines high performance liquid chromatography (HPLC) and mass spectrometry. Compounds of high molecular weight or temperature sensitivity can be separated before proceeding to the MS. Detection is often performed by UV- or fluorescence-detectors. HPLC is not only used for analysis, but also to purify substances. The mobile phase with the sample passes a column, the stationary phase, which is coated inside with an unpolar layer. Depending on their polarity molecules inside the mobile phase are retained to a different degree. Apolar molecules are retarded and exit the column later. Often a pre-column is installed to reduce contamination. LC-MS is less effective then GC-MS in discriminating between substances with similar retention time.

Mass spectrometry

By mass spectrometry (MS) the mass-charge-ratio (m/q) of ions is measured. It is possible to calculate masses of ions with known charge and to detect and count ions with known m/q-ratio. The method can be used to analyze and/or characterize the structure, composition or quantity of a substance (metabolite or protein) in a sample.

A mass spectrometry means two steps – ionization and mass-detection.

The **ion source** ionizes the molecules. For this purpose, multiple tools have been developed. Electron Impact (EI), Electron-spray ionization (ESI), Chemical ionization (CI), Atmospheric pressure photoionization (APPI), Liquid injection field desorption ionization (LIFDI), or Matrix-assisted laser desorption/ ionization (MALDI). For EI gases or evaporated liquids are pounced against each other to produce ions, which are often instable. LIFDI works with pressure differences between vacuum and normal pressure. The liquid sample is injected through a capillary, which makes it an appropriate method especially for air-sensitive substances. Polar or charged substance mixes can be measured best with ESI. Ionized

metabolites are dissolved in samples, formed into small droplets and sprayed into a vacuum. Only the analyte persists and can be measured. ESI is an adequate method for measuring big molecules or proteins, typically used in LC. Alternatively APPI is used with the advantage of processing also less polar molecules which cannot be ionized with other methods (Kopka *et al.*, 2004). MALDI uses dried samples, mixed with a large amount of "matrix" and crystallized. A laser dissolves the analyte. ESI, CI and APPI are so-called soft ionization methods, working with less energy and producing molecules with a lower affinity to form fragments (Kopka *et al.*, 2004).

Mass detection separates ions according to their m/q. The resolution differs according to the methods. Three main technologies are used to detect and quantify ions – ion-trap (TRAP), quadrupole detectors (QUAD) and time-of-flight (TOF).

QUAD works with metal rods operating as mass-selective ion filters counting the ions which pass the filter. A variation are triple quadrupole detectors, consisting of three quadrupole detectors in a row and refining the detection. Methods using TRAP trigger incoming ions into a stable orbit. According to the mass, ions leave the orbit after a certain time and are counted. It is possible to collect and store these ions to apply another round of fragmentation which enables the determination of molecular structures. Finally TOF is used for macromolecules, like proteins, polysaccharides and other polymers. Ions are moving in groups and are measured at a fixed point. Speed and arrival vary according to the mass of an ion. This information is used for characterization (Kopka *et al.*, 2004, 2005).

2.5. The Aim of this work

The experiments and results described here were performed to gain new insights into the field of heterosis. As described before, hybrid vigor is a phenomenon affecting many important traits. It is one of the major possibilities to enhance plant tolerance against abiotic stresses and the molecular basis is still unknown. The identification of the roots of this phenomenon would be ecologically and economically very important.

Abiotic stresses are multigenic. To find and modify all involved genes to produce a plant tolerant to a certain stress would be very time consuming and most likely practically impossible. The newly achieved combination of allels for several traits in the heterozygous hybrid leads to an enhancement compared to the parental lines. Heterosis is the result of crossing which is faster and results in uniform naturally enhanced individuals (F1). Freezing tolerance is an easily measurable trait of high agricultural importance.

Freezing tolerance of all F1 - hybrids and the content of five compatible solutes (glucose, fructose, sucrose, raffinose and proline) was measured was determined accumulation in non acclimated (NA) plants and after cold acclimation at 4°C (ACC). From the results absolute heterosis for all accessions and substances under both conditions (NA, ACC) was calculated. Based on the strength of hybrid vigor detected, eight crosses were chosen for further investigation by global metabolite profiling by GC-MS and the detection of flavonoids by LC-MS.

Freezing tolerance, sugar and proline measurements have been repeated for F2- hybrids to evaluate the assumed decrease of heterosis with increasing homozygosity. Rohde *et al.* (2004) have shown a loss of heterosis in reciprocal F2 crosses of the *Arabidopsis thaliana* accessions C24 and Columbia (Col-0).

My main interest was to investigate the influence of heterosis on metabolites and metabolic changes in freezing tolerance in the context of enhanced freezing tolerance in the hybrids compared to the parental lines.

Do all investigated compounds show heterotic effects in all crosses? Does the strength and occurrence of heterosis vary between the metabolites, accessions and under both conditions? Furthermore we were interested in the occurrence of maternal affects in reciprocal crosses. An important part of my work was to look for correlations between freezing tolerance and metabolite content, but also between Heterosis in freezing tolerance and Heterosis in metabolite content.

To explore this, I compared the occurrence and strength of freezing tolerance and cold related metabolite performance under two conditions (NA, ACC) in three generations (P, F1, F2).

3. Paper 1 (P1)

Heterosis in the freezing tolerance, and sugar and flavonoid contents of crosses between *Arabidopsis thaliana* accessions of widely varying freezing tolerance

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Authors contribution

The research was conceived and planned by Dirk K. Hincha and Marina Korn. The experimental work was done by Marina Korn. Flavonoid measurement was done by Silke Peterek but evaluated by Marina Korn.

3.1. Abstract

Heterosis is defined as the increased vigour of hybrids in comparison to their parents. We investigated 24 F1 hybrid lines of *Arabidopsis thaliana* generated by reciprocally crossing either C24 or Col with six other parental accessions (Can, Co, Cvi, L*er*, Rsch, Te) that differ widely in their freezing tolerance. The crosses differed in the degree of heterosis for freezing tolerance, both in the non-acclimated state and after a 14 d cold acclimation period. Crosses with C24 showed more heterosis than crosses with Col, and heterosis was stronger in acclimated than in non-acclimated plants. Leaf content of soluble sugars and proline showed more deviation from mid-parent values in crosses involving C24 than in those involving Col, and deviations were larger in acclimated than in non-acclimated plants. There were significant correlations between the content of different sugars and leaf freezing tolerance, as well as between heterosis effects in freezing tolerance and sugar content. Flavonoid content and composition varied between accessions, and between non-acclimated and acclimated plants. In the crosses, large deviations from the mid-parent values in the contents of different flavonols occurred, and there were strikingly strong correlations between both flavonol content and freezing tolerance, and between heterosis effects in freezing tolerance and flavonol content.

Key-words: cold acclimation; compatible solutes.

3.2. Introduction

Freezing tolerance is a primary factor that defines the geographic distribution of plants. In addition, it has a strong influence on the yield of crop plants in large parts of the world, where frost can lead to periodic catastrophic yield losses. Many plants from temperate and cold climates, including many important crop species, increase in freezing tolerance during exposure to low, but non-freezing temperatures in a process termed cold acclimation (see Thomashow 1999; Xin & Browse 2000; Smallwood & Bowles 2002 for comprehensive reviews). To understand the molecular basis of plant freezing tolerance, a strong emphasis has been on molecular analyses in the model plant *Arabidopsis thaliana*.

Plant freezing tolerance is a multigenic, quantitative trait, and gene expression profiling with whole genome arrays indicates that cold acclimation in *A. thaliana* involves changes in the expression levels of several hundred genes (Hannah, Heyer & Hincha 2005; Vogel *et al.* 2005; Hannah *et al.* 2006; Kaplan *et al.* 2007), while metabolite profiling revealed changes in

the content of a large portion of cellular metabolites (Cook *et al.* 2004; Kaplan *et al*. 2004, 2007; Hannah *et al.* 2006).While many of these changes are probably associated with specific adaptations in metabolic pathways to low growth temperature (Guy *et al.* 2008), at least some metabolites may also function as compatible solutes.

Compatible solutes are synthesized by many organisms ranging from bacteria to animals and plants, in response to desiccation, osmotic stress, salt stress or low temperature. This chemically heterogeneous group of substances comprises some amino acids (e.g. proline), quaternary ammonium compounds (e.g. betaine), many sugars, sugar alcohols and several others (see Yancey *et al.* 1982; Somero 1992 for reviews). Physiologically, compatible solutes should have no adverse metabolic effects even at very high concentrations. They are thought to stabilize sensitive cellular components under stress conditions and also act as bulk osmoprotectants. Therefore, they may act colligatively by increasing the osmotic potential and thereby improving the water status, and therefore, the cell volume in the frozen state. In addition, they can stabilize macromolecular structures such as proteins by preferential exclusion from the hydration shell of proteins (Timasheff 1993), assist refolding of unfolded polypeptides by chaperone proteins (Diamant *et al.* 2001) and stabilize membranes during freezing and drying (Crowe *et al.* 1990; Hincha, Popova & Cacela 2006). However, the contribution of different compatible solutes such as sugars and proline to the freezing tolerance and cold acclimation of *Arabidopsis* and other plant species has not yet been resolved (see Hincha *et al.* 2005 for a recent review).

Attempts to understand the genetic and molecular basis of complex quantitative traits in plants have in recent years focused on the analysis of natural genetic variation. *Arabidopsis thaliana* is a geographically widely spread species, and it has been shown that different accessions have sufficient genetic variability to allow investigations of genotype x environment interactions (see Koornneef, Alonso-Blanco & Vreugdenhil 2004; Mitchell-Olds & Schmitt 2006 for reviews). This is also true for freezing tolerance, where a clear correlation with both latitude of origin and habitat growth temperature has been shown (Hannah *et al.* 2006; Zhen & Ungerer 2008). Recently, quantitative trait locus (QTL) mapping was successfully employed to gain insight into the molecular basis of the differences in acclimated freezing tolerance between the accessions Cape Verde Islands (Cvi) and Landsberg *erecta* (L*er*) (Alonso-Blanco *et al.* 2005). In addition, crosses of *Arabidopsis* accessions can be used to create further genetic and phenotypic variation through heterosis effects (see e.g. Griffing & Scholl 1991; Narang & Altmann 2001; Barth *et al.* 2003; Meyer *et al.*2004).

The analysis of such heterotic offspring could lead to new insights into the molecular basis of plant freezing tolerance. In addition, in an almost exclusively selfing species like *A.thaliana* (Abbott & Gomes 1989), accessions are largely homozygous, and this is expected to lead to inbreeding depression. Crossing such accessions leads to increased heterozygosity,

which may result in heterosis. The term heterosis (Shull 1914) describes the phenomenon of increased physiological performance of F1 hybrids in comparison to their parents. This can be expressed either as mid-parent heterosis (MPH), or best-parent heterosis (BPH). MPH denotes the deviation of the F1 from the parental mean and can be either positive or negative. BPH denotes cases where the F1 outperforms the better parent and can, by definition, only be positive. Although heterosis has been used extensively by breeders to increase the performance of crop plants, our understanding of its molecular basis is still rudimentary (see Birchler, Auger & Riddle 2003; Hochholdinger & Hoecker 2007 for reviews).

In a recent publication, we have shown that both MPH and BPH can be observed in the freezing tolerance of reciprocal crosses of the *Arabidopsis* accessions C24 and Col (Rohde, Hincha & Heyer 2004). Here, we demonstrate that heterosis in *Arabidopsis* freezing tolerance is not restricted to this combination of genotypes, but that it occurred in a wide range of crosses involving C24, while the heterosis effects were generally smaller in crosses involving Col. There were also large deviations from mid-parent values in the content of several sugars and in the content of different flavonols. The content of some, but not all, of these compounds correlated strongly with freezing tolerance in the accessions and crosses. In addition, we showed for the first time strong correlations between the heterosis effects in freezing tolerance and the content of some of these compounds, suggesting possible causative relationships.

3.3. Materials and methods

Plant material

We used *A. thaliana* plants from the accessions C24, Canary Islands (Can), Coimbra-2 (Co), Columbia-0 (Col), Cape Verde Islands (Cvi), Landsberg *erecta* (L*er*), Rschew (Rsch) andTenela (Te). The sources of the different seed stocks have been described in a recent publication (Schmid *et al.* 2006). Seeds for our experiments have been generated through single seed descent to assure genetic homogeneity of the plants (Törjek *et al.* 2003). Plants were grown in soil in a greenhouse at 16 h day length with light supplementation to reach at least 200 mEm–2 s–1 and a temperature of 20 °C during the day, 18 °C during the night until bolting (compare Hannah *et al.* 2006). For cold acclimation, plants were transferred to a 4 °C growth cabinet at 16 h day length with 90 mEm–2 s–1 for an additional 14 d. The reduced light intensity avoids the risk of photoinhibitory damage at the low temperature, but is sufficient to allow cold acclimation, including the accumulation of sugars and starch (Rohde *et al.* 2004; Zuther *et al.* 2004; Hannah *et al.* 2006; Guy *et al.*2008).

Freezing experiments

Freezing damage was determined as electrolyte leakage after freezing of detached leaves to different temperatures as described in detail in previous publications (Rohde *et al.*2004; Hannah *et al.* 2006). Between 12 and 24 plants were analyzed in each experiment from each genotype and treatment. All experiments were performed at least twice. The LT_{50} was calculated as the LOGEC50 value of unanchored sigmoidal curves fitted to the leakage values using the software GraphPad Prism3. Statistical analysis of the differences in LT_{50} values was performed using *t*-tests with GraphPad Instat software. Analyses of variance (anovas) were performed with the statistics package in the Kaleida-Graph software (Synergy Software, Reading, PA, USA).

Carbohydrate analysis

Three leaves from individual plants that were also used in the freezing experiments were harvested at midday (6–8 h after lights on). They were frozen in liquid nitrogen immediately after sampling and homogenized using a ball mill Retsch MM 200 (Retsch, Haan, Germany). Soluble sugars were extracted and analysed by high-performance liquid chromatography (HPLC) as described previously (Rohde *et al.* 2004). Statistical analysis was performed using *t*-tests in Excel.

Proline measurements

Proline measurements were performed photometrically as previously described (Bates, Waldren & Teare 1973; Rohde *et al.* 2004) on the same leaf samples that were also used for sugar measurements. Statistical analysis was performed using *t*-tests in Excel.

Analysis of flavonoids by liquid chromatography–mass spectrometry (LC–MS)

Powdered plant material was extracted twice with 80% methanol at a ratio of 1:8 (leaf fresh weight : volume) and cleared by centrifugation. Phenylpropanoids were separated by reversed-phase HPLC (Acquity UPLC with a BEH C18, 1.7 mm, 2.1 x 50 mm column; Waters, Eschborn, Germany) at 25 °C. The mobile phase was composed of 98% water, 2% formic solution (= 5% ammonium formate in formic acid) (solvent A) and 100% acetonitrile (solvent B). Flavonoids were eluted with the following gradient at a flow rate of 0.6 mL min–1: initial, 0% B; 1.0 min, 0% B; 3.5 min, 40% B; 4.0 min, 100% B; 4.25 min, 100% B; 4.5 min, 0% B; 5.0 min, 0% B.

Eluted substances were detected with a photodiode array detector (PDA 2996; Waters).Ten absorbance spectra were recorded every second, between 210 and 600 nm, with a bandwidth of 1.2 nm, and chromatograms were extracted from the PDA data at 280 nm. Data were analyzed using Waters MassLynx software.

The eluted substances were identified using an electrospray ionization–time-of-flight (ESI–ToF) mass spectrometer (LCT Premier; Waters). The mass spectrometer operated in a positive ion mode with a source temperature of 100 °C and a cone gas flow of 5.0 L h–1. The mass spectra were acquired with the mass analyzer in W-mode, and spectra were integrated over 1 s intervals. MS data were acquired in a continuum mode using MassLynx 4.1 software (Waters). The instrument was calibrated with a multi-point calibration using selected fragment ions of phosphoric acid. Leucine enkephalin was used as lockspray reference probe.

3.4. Results

Heterosis in the freezing tolerance of crosses between *Arabidopsis* accessions

We determined the freezing tolerance of *Arabidopsis* by slowly freezing detached leaves to different temperatures. After thawing, electrolyte leakage was determined with a conductimeter, and from the fitted sigmoidal curves the LT_{50} (temperature of 50% electrolyte leakage) was calculated as a quantitative measure of leaf freezing tolerance (Rohde *et al.* 2004; Hannah *et al.* 2006).Figure 1 shows as an example the electrolyte leakage curves for non-acclimated plants (NA) of the accessions C24 and Co, and of one of the reciprocal crosses (C24 x Co). As noted before (Hannah *et al.* 2006), Co was more freezing tolerant than C24 (LT_{50} = –5.4 and –4.0 °C, respectively). The F1 cross of these two parental accessions was more freezing tolerant than either parent (LT_{50} = –6.3 °C), resulting in significant MPH (27%) and BPH (23%), similar to the situation previously reported for C24 and Col (Rohde *et al.* 2004).

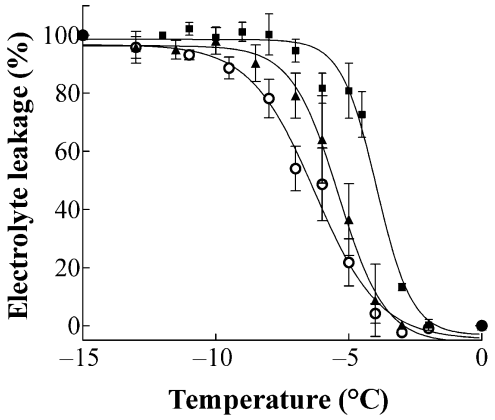

Figure 1. Electrolyte leakage from *Arabidopsis thaliana* leaves after freezing and thawing. Leaves from non-acclimated plants of the accessions C24 (solid squares) and Co (solid triangles), and a cross between the two accessions (C24 x Co; open circles) were frozen to different temperatures. The electrolyte leakage values are the means ± standard error of the mean (SEM) from three measurements. Sigmoidal curves were fitted to the data to determine the LT_{50} values (temperature of 50% electrolyte leakage), which were –4.0 ± 0.15 °C for C24, –5.4 ± 0.22 °C for Co and –6.3 ± 0.22 °C for the C24 x Co F1 plants.

It is generally not clear which parental properties, such as variation in parental phenotypical or genetical differences, determine the magnitude of heterotic effects. Therefore, we performed systematic manual pollination experiments to cross the already characterized accessions C24 and Col with a panel of six accessions varying widely in freezing tolerance (non-acclimated LT_{50} between –4.5 °C for C24 and –7.1 °C for Te; acclimated LT_{50} between –6.2 °C for Co and Can, and –12.1 °C for Te; Hannah *et al.* 2006) and geographic origin (16° to 66° northern latitude; Hannah *et al.* 2006). For all reciprocal crosses and their parents, freezing experiments were performed as described in Fig. 1, both before and after cold acclimation for 14 d at 4 °C. Figure 2 shows the heterosis effect on freezing tolerance for all 28 crosses, including the previously published data (Rohde *et al.* 2004) for C24 x Col and Col x C24 for comparison. Statistical analysis showed a highly significant influence of genotype on the magnitude of the heterosis effect ($F = 4.59$; $P < 0.0001$; Fig. 2), validating this approach.

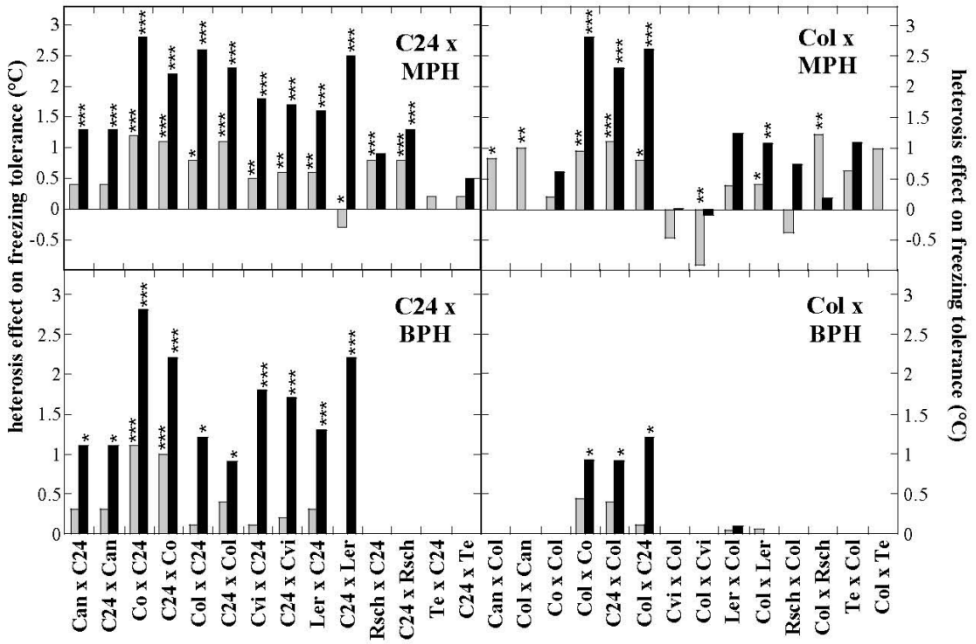

Figure 2. Heterosis effects on the freezing tolerance of leaves from F1 plants generated by systematically crossing the accessions C24 and Col with a panel of accessions varying in freezing tolerance. Leaves were harvested either from non-acclimated (grey bars) or cold-acclimated (black bars) plants. Heterosis was calculated either as mid-parent heterosis (MPH) or as best-parent heterosis (BPH). Analysis of variance (anova) analysis showed a significant influence of genotype on heterosis (F = 5.04; P <0.0001), and a significant effect of cold acclimation on both MPH (F = 19.77; $P \leq 0.0001$) and BPH (F = 12.10; P = 0.0011). The significance of the heterosis effects in the different F1 populations was determined by t-test and is indicated by the asterisks above the bars (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). The data for the C24 x Col and Col x C24 plants were taken from Rohde *et al.* (2004).

Two conclusions are immediately apparent from these data. Heterosis was always larger in acclimated than in non-acclimated plants, and in general heterosis effects were bigger and more frequent in crosses involving C24 than in crosses involving Col. The highest relative MPH and BPH were found in Co x C24 with 27 and 23% for nonacclimated plants, and with 44% for both MPH and BPH for acclimated plants. Crosses involving Col reached a maximum of 18% MPH in the non-acclimated state (Col x Co) and 31% (Col x Co) in the acclimated state. Significant BPH was only observed after cold acclimation and reached a maximum of 23% in Col x C24.

While in most combinations of accessions the two reciprocal crosses showed very similar levels of heterosis, there were some remarkable exceptions. The two most striking ones are the combinations of C24 and L*er*, and of Col and Co (Fig. 2). Interestingly, these presumed maternal effects could also depend on the paternal genotype, as the reciprocal combinations of Col and L*er* showed no corresponding differences.

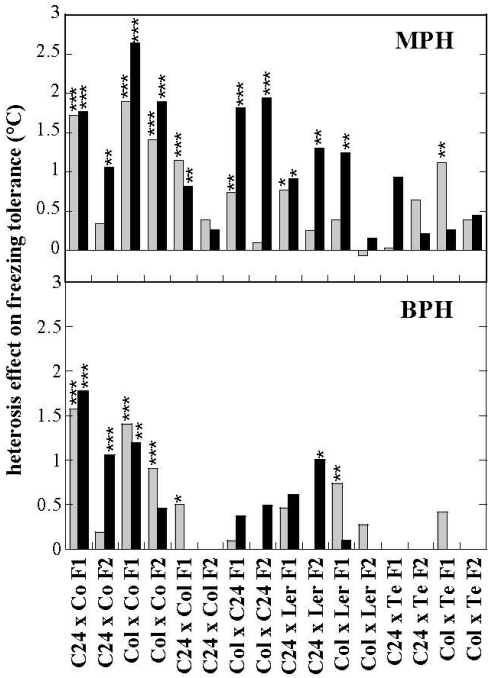

Figure 3. Heterosis effects on the freezing tolerance of leaves from F1 plants generated by crossing the accessions C24 and Col with a subset of the accessions shown in Fig. 2 and the respective F2 plants generated by selfing the F1 plants. Leaves were harvested either from non-acclimated (grey bars) or cold-acclimated (black bars) plants. Heterosis was calculated either as mid-parent heterosis (MPH) or as best-parent heterosis (BPH). The significance of the heterosis effects was determined by *t*-test and is indicated by the asterisks above the bars (compare Fig. 2).

Because heterotic effects are by definition (Shull 1914) based on heterozygosity in the F1 plants, further inbreeding should reduce heterosis. For a selected panel of crosses, we have therefore obtained F2 plants by selfing the respective F1 plants and compared the heterotic effects on freezing tolerance (Fig. 3). In most cases, a reduction in heterosis was apparent. This indicates that the F2 populations showed segregation towards transgression to less freezing-tolerant phenotypes. However, in C24 x L*er*, heterosis of acclimated plants increased in the F2 compared to the F1. This is not related to the maternal effect observed in this cross, because the C24 x Co cross showed the expected reduction of heterosis in the F2 (Fig. 3). It is therefore possible that the heterosis in the C24 x L*er* cross is caused by the stable complementation of a defective gene(s) rather than increased heterozygosity.

Compatible solutes in the different genotypes

During plant cold acclimation, the content of most solutes in leaf cells increases (Cook *et al.* 2004; Kaplan *et al*. 2004, 2007; Hannah *et al.* 2006), and there is evidence from several studies that at least some of these solutes may be important for the development of freezing tolerance (see Xin & Browse 2000; Smallwood & Bowles 2002 for reviews).We have

therefore measured the amounts of four sugars (Fru, Glc, Suc,Raf) and the amino acid proline in leaf samples from all parental accessions and their reciprocal F1 hybrids, both before and after cold acclimation.

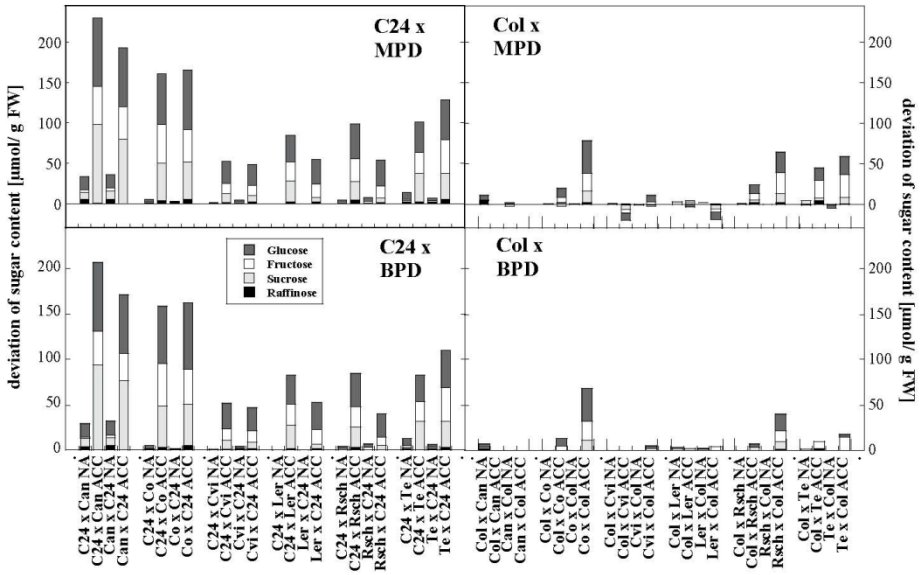

Figure 4. Deviations from mid-parent or best-parent values in the content of soluble carbohydrates in the leaves of the F1 plants characterized for freezing tolerance in Fig. 2. Leaves were harvested either before (NA) or after (ACC) cold acclimation. Analysis of variance (anova) showed a significant influence of genotype on heterosis for all sugars (Glc: $F = 77.11$; Fru: $F = 36.99$; Suc: $F = 618.43$; Raf: $F = 16.74$; $P < 0.0001$ for all sugars). The statistical significance of all deviations in sugar content is shown in Supplementary Table S1.

The data were used to calculate deviations from midparent and best-parent values (i.e. heterosis effects) in the sugar content of all F1 plants investigated in this study (i.e. not for the crosses between C24 and Col, where this has been published previously; Rohde *et al.* (2004). Because the term heterosis is used in the context of traits like seed yield, biomass or stress tolerance (Hochholdinger & Hoecker 2007), we used the terms mid-parent deviation (MPD) and best-parent deviation (BPD) in the remainder of this paper for the content of different substances in the plants. They were calculated identically to MPH and BPH in freezing tolerance, and therefore allow quantitative comparisons. We found deviations from both mid-parent and best-parent values for the content of all four sugars (Fig. 4) in a pattern similar to that obtained for the heterosis in freezing tolerance (Fig. 2). These deviations in sugar content depended significantly on genotype and were much larger and more frequent in cold-acclimated than in non-acclimated plants. Furthermore, the effects were stronger and more frequent in crosses involving C24 than in those involving Col. Interestingly, the differences in heterosis effects between some reciprocal crosses observed for freezing tolerance described

earlier were not reflected in the corresponding sugar contents. Supplementary Table S1 gives an overview of the statistical significance of all MPD and BPD values.

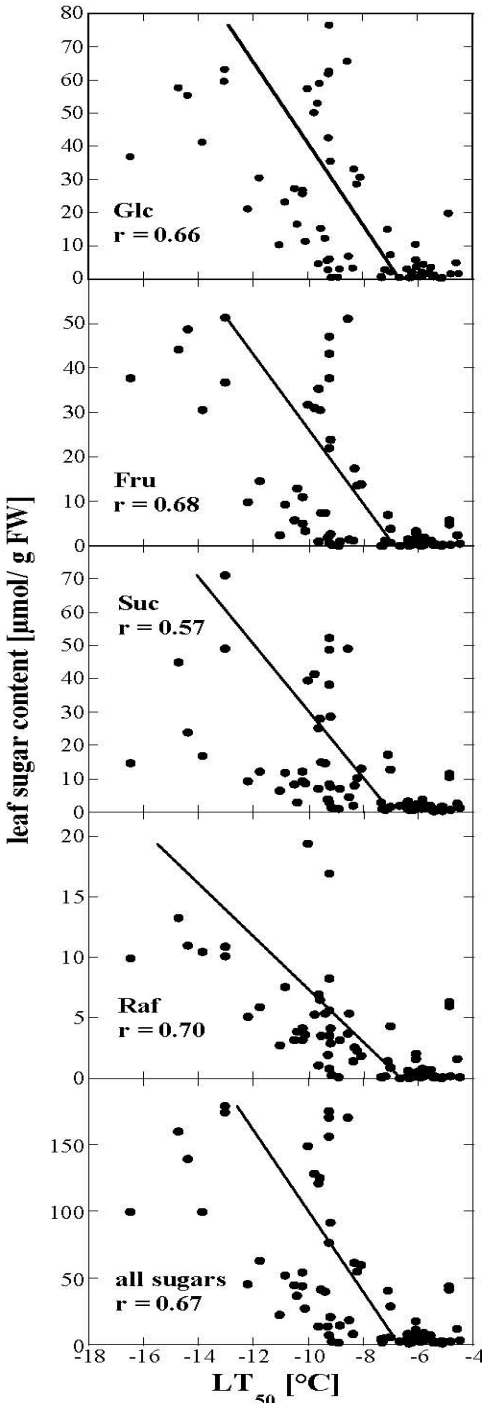

Figure 5. Analysis of the correlations between the content of different soluble sugars in the leaves and leaf freezing tolerance. Data were compiled from all investigated accessions and their F1 for both non-acclimated and cold-acclimated plants. For the bottom panel (all sugars), the content of Glc, Fru, Suc and Raf was added up for each sample. The lines were fitted to the data by least square linear regression analysis, and the correlation coefficients are shown in the panels. The *P* values for all correlations were below 0.0001.

To obtain insight into the functional significance of the accumulated sugars in freezing tolerance as such and in heterosis for freezing tolerance, we first correlated sugar content with LT50 (Fig. 5). The content of all four sugars showed a relatively good correlation with LT_{50} (from $r = 0.57$ for sucrose to $r = 0.70$ for raffinose; all P values below 0.0001). The correlation was of similar quality ($r = 0.67$) when the content of all sugars was added up (bottom panel in Fig. 5), indicating that the individual sugars may not play highly specific roles in leaf freezing tolerance.

Similarly, the role of differences in sugar content in the establishment of heterosis in freezing tolerance was evaluated by correlating heterosis effects in LT_{50} with the respective MPD and BPD for sugar content, separately for MPH and BPH (Fig. 6). In general, these correlations were weaker than those between LT_{50} and sugar content, as indicated by both the r and P values (compare Fig. 5), and they were better for BPH than for MPH. MPD and BPD in sucrose content showed the lowest correlations with heterosis in freezing tolerance, and MPD in raffinose content also showed a surprisingly low correlation. This correlation, however, was much better when BPD was considered. For both MPD and BPD, the correlations obtained after adding up the effects of all sugars were within the range of the correlation coefficients for the separate effects of the individual sugars, again suggesting redundancy between the sugars.

Another potentially beneficial solute that *Arabidopsis* accumulates during cold acclimation is proline. Figure 7 shows that deviations from mid-parent or best-parent values were less frequent for proline than for sugar content (compare Fig. 4), but still depended significantly on genotype. Several of the F1 plants derived from crosses with Col showed strong negative MPD. Consequently, while there was a moderate, but significant correlation between LT_{50} and proline content of the investigated genotypes (Fig. 8), there was no significant correlation between either MPD or BPD in proline content, and MPH or BPH in freezing tolerance (data not shown), indicating that differential proline accumulation played no major role in the establishment of heterosis effects in *Arabidopsis* freezing tolerance.

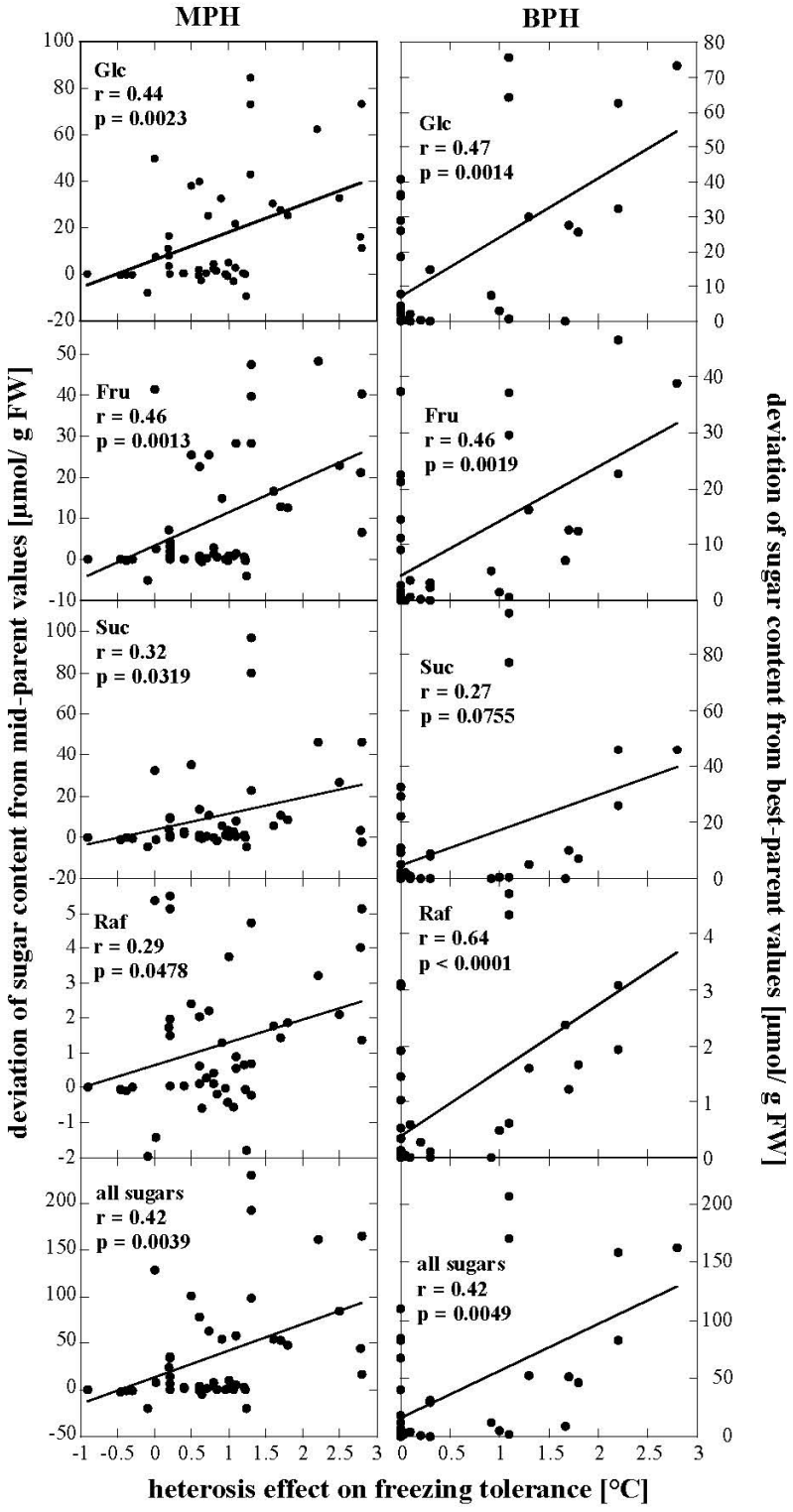

Figure 6. Analysis of the correlations between deviations from mid-parent or best-parent values in the content of different soluble sugars in the leaves and the heterosis effects on leaf freezing tolerance (compare Figs 2 & 4). Data were compiled from all investigated accessions and their F1 for both non-acclimated and cold-acclimated plants, and include both statistically significant and non-significant effects. The lines were fitted to the data by least square linear regression analysis, and the correlation coefficients and *P* values are shown in the panels. Analyses were performed for both mid-parent heterosis (MPH, left panels) and best-parent heterosis (BPH, right panels) in freezing tolerance.

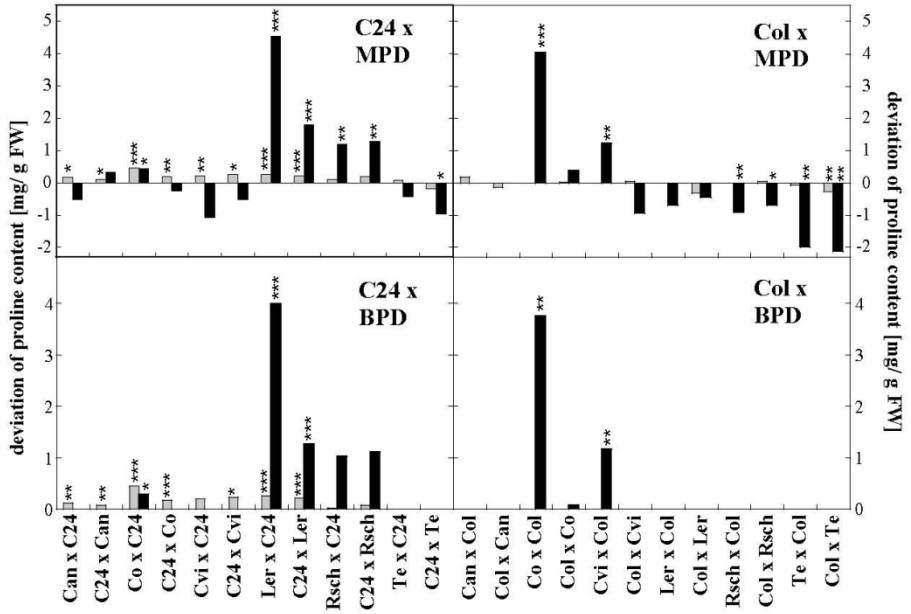

Figure 7. Deviation from mid-parent (MPD) and best-parent (BPD) values of proline content in the leaves of the F1 plants characterized for freezing tolerance in Fig. 2. Leaves were harvested either before (grey bars; NA) or after (black bars; ACC) cold acclimation. Analysis of variance (anova) showed a significant influence of genotype on heterosis ($F = 2.90$; $P = 0.00077$). Significance of the heterosis effect in the different F1 populations was determined by *t*-test and is indicated by the asterisks above the bars (compare Fig. 2).

Flavonoid content and composition in the different genotypes

One of the major biochemical pathways in *Arabidopsis* that is strongly cold induced at the transcript level is flavonoid biosynthesis (Hannah *et al*. 2005, 2006). We used LC–MS technology to comprehensively profile the flavonoid composition of a subset of five accessions and eight F1 crosses, both before and after cold acclimation. This subset of accessions and crosses covers the whole range of LT_{50} values and heterosis effects in LT_{50} determined in the larger selection described earlier.

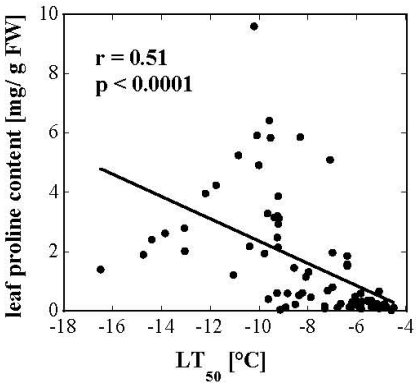

Figure 8. Analysis of the correlation between the proline content of *Arabidopsis* leaves and leaf freezing tolerance. Data were compiled from all investigated accessions and their F1 for both non-acclimated and cold-acclimated plants. The line was fitted to the data by least square linear regression analysis, and the correlation coefficient and *P* value are shown in the panel.

Figure 9 shows the HPLC elution profiles of flavonoids extracted from the leaves of the accessions C24 and Te, which constitute the extremes in freezing tolerance in our selection of accessions (compare Hannah *et al.* 2006). Comparison of the four upper panels (a1/b1; a2/b2) with the four lower panels (a3/b3; a4/b4) reveals that the more freezing tolerant accession Te contained higher amounts of flavonoids than the less freezing-tolerant accession C24. However, both accessions showed a clear increase in flavonoid content with cold acclimation (compare e.g. a1 with a2, and a3 with a4).An integration of the peak areas yielded quantitative data for further analysis.

The substances detected at 280 nm were characterized by absorbance spectroscopy and mass spectrometry. Ten peaks could be clearly resolved by HPLC (Fig. 9, b3). Peaks 3 and 7 were not further characterized, as they contained anthocyanins that could only be detected in extracts from cold-acclimated leaves from Te. Absorbance spectroscopy indicated that the minor peaks 9 and 10 contained cinnamic acid derivatives that were also not further characterized. Peaks 1, 2, 4, 5, 6 and 8 contained flavonol derivatives. Peaks 1 and 2 contained quercetin as the aglycon with different numbers of Glc and Rha molecules attached, while peaks 5, 6 and 8 contained kaempferol as the aglycon, and also Glc and Rha as sugar substituents (Table 1). For the quantitative analysis of leaf flavonoids, we only used the major peaks 2, 4, 6 and 8.

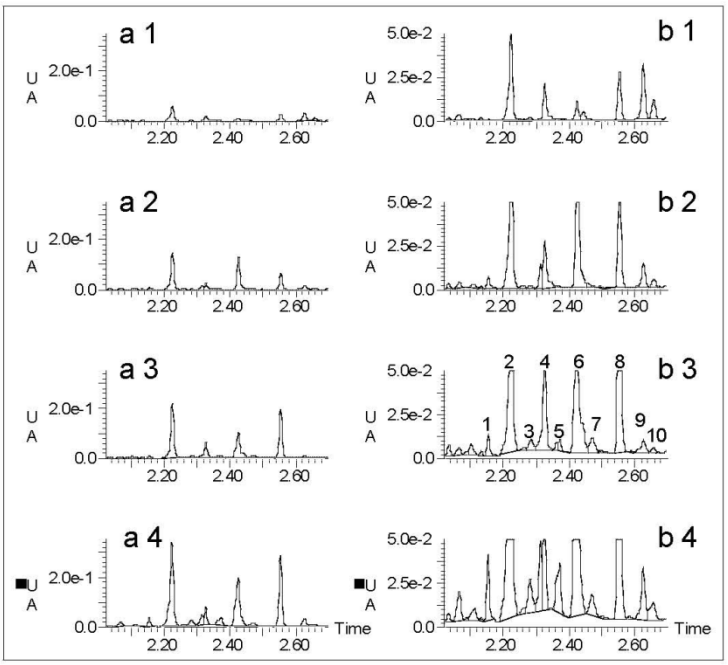

Figure 9. High-performance liquid chromatography (HPLC) analysis of flavonoids from leaves of the *Arabidopsis* accessions C24 (a1/a2 and b1/b2) and Te (a3/a4 and b3/b4) before (a1/b1 and a3/b3) and after (a2/b2 and a4/b4) cold acclimation. Absorbance spectra were taken with a diode array detector, and absorbance at 280 nm is plotted in the figure. In addition, the peaks numbered 1–10 in panel b3 were analysed by mass spectroscopy, and both absorbance and mass spectra were used to determine the substances underlying the peaks (see Table 1). The areas of peaks 2, 4, 6 and 8 were quantified to compare flavonoid content and composition (Figs 10–12). Right panels (b1–b4) are enlargements of the chromatograms shown in the corresponding left panels (a1–a4), revealing changes also for minor components.

Several different F1 plants showed significant deviations from mid-parent or best-parent values in their content of various flavonols (Fig. 10), and the magnitude of both MPD and BPD for all flavonols depended significantly on plant genotype. Only the C24 x Te cross displayed strongly negative MPD, while the Col x Te cross showed positive MPD and also BPD. The crosses of C24 and Col with L*er* yielded no significant deviations for any flavonols (Supplementary Table S2), while the reciprocal crosses between C24 and Col, as well as the C24 and Col crosses with Co showed MPD and BPD.

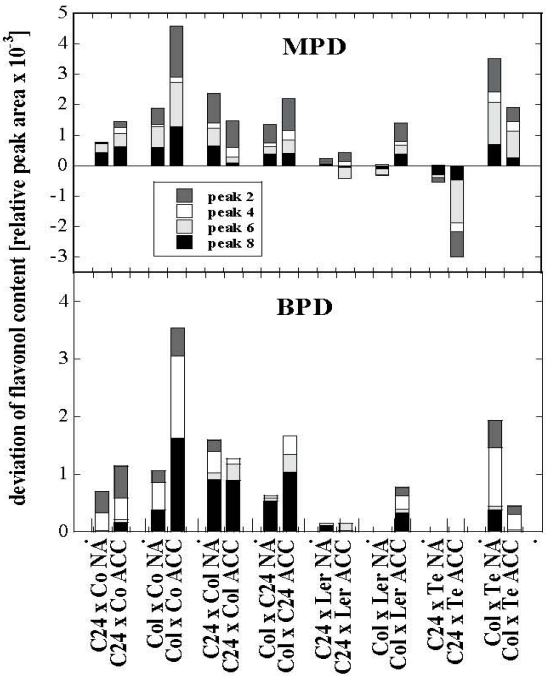

Figure 10. Deviation from mid-parent (MPD) and best-parent (BPD) values of the content of different flavonol glycosides in the leaves of selected *Arabidopsis* crosses. Leaves were harvested either before (NA) or after (ACC) cold acclimation. The peak numbering refers to the high-performance liquid chromatography (HPLC) elution profile shown in Fig. 9, b3. Further information about the analysed compounds can be found in Table 1. Analysis of variance (anova) showed a significant influence of genotype on heterosis for all flavonols (peak 2: $F = 0.21$, $P < 0.0001$; peak 4: $F = 3.74$,

$P = 0.014$; peak 6: $F = 5.55$, $P = 0.00098$; peak 8: $F = 3.83$, $P = 0.0067$). The statistical significance of all deviations in flavonol content is shown in Supplementary Table S2.

The amounts of flavonols in the major peaks and the sum of all flavonols in these peaks were strongly (r values between 0.83 and 0.90; all P values below 0.0001) correlated with leaf freezing tolerance (Fig. 11). The correlations between heterosis in leaf freezing tolerance and deviations in flavonol content from mid-parent and best-parent values indicated specific roles for particular flavonols (Fig. 12).

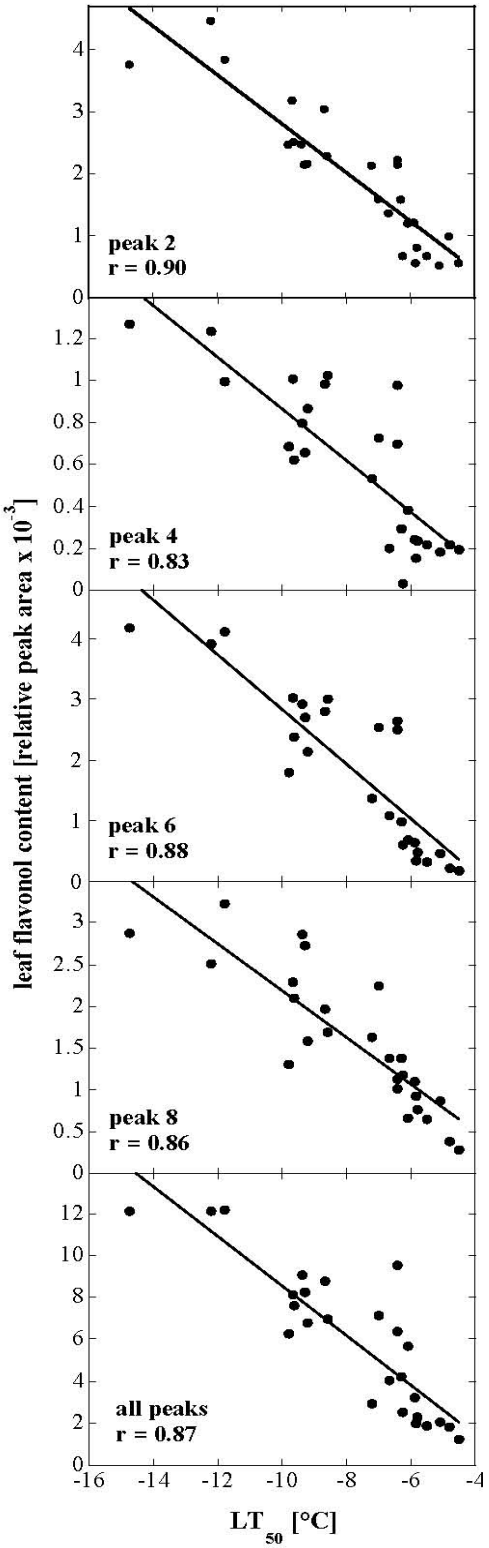

Figure 11. Analysis of the correlations between the flavonol content of *Arabidopsis* leaves and leaf freezing tolerance. Data were compiled from all investigated accessions and their F1 for both non-acclimated and cold-acclimated plants. For the bottom panel (all flavonols), the content of all four flavonol peaks (peaks 2, 4, 6 and 8; compare Fig. 9 and Table 1) was added up for each sample. The lines were fitted to the data by least square linear regression analysis, and the correlation coefficients are shown in the panels. The *P* values for all correlations were below 0.0001.

MPD and BPD in the content of quercetin glycosides (peak 4) showed no significant correlation with heterosis in freezing tolerance. The same was true for BPD in the content of the flavonol derivatives contained in peak 2, but not for MPD. The highest correlations, however, were found for the MPD and BPD of the kaempferol glycosides in peak 8 (r = 0.77 and 0.75, respectively). These correlations were much better than those obtained when the MPD or BPD of all flavonols was added up (r = 0.55 and 0.39), or those for peak 6, which contained a different kaempferol glycoside and Rha as sugar substituents (Table 1).

For the quantitative analysis of leaf flavonoids, we only used the major peaks 2, 4, 6 and 8. Several different F1 plants showed significant deviations from mid-parent or best-parent values in their content of various flavonols (Fig. 10), and the magnitude of both MPD and BPD for all flavonols depended significantly on plant genotype. Only the C24 x Te cross displayed strongly negative MPD, while the Col x Te cross showed positive MPD and also BPD. The crosses of C24 and Col with L*er* yielded no significant deviations for any flavonols (Supplementary Table S2), while the reciprocal crosses between C24 and Col, as well as the C24 and Col crosses with Co showed MPD and BPD. The amounts of flavonols in the major peaks and the sum of all flavonols in these peaks were strongly (r values between 0.83 and 0.90; all P values below 0.0001) correlated with leaf freezing tolerance (Fig. 11). The correlations between heterosis in leaf freezing tolerance and deviations in flavonol content from mid-parent and best-parent values indicated specific roles for particular flavonols (Fig. 12). MPD and BPD in the content of quercetin glycosides (peak 4) showed no significant correlation with heterosis in freezing tolerance. The same was true for BPD in the content of the flavonol derivatives contained in peak 2, but not for MPD. The highest correlations, however, were found for the MPD and BPD of the kaempferol glycosides in peak 8 (r = 0.77 and 0.75, respectively). These correlations were much better than those obtained when the MPD or BPD of all flavonols was added up (r = 0.55 and 0.39), or those for peak 6, which contained a different kaempferol glycoside.

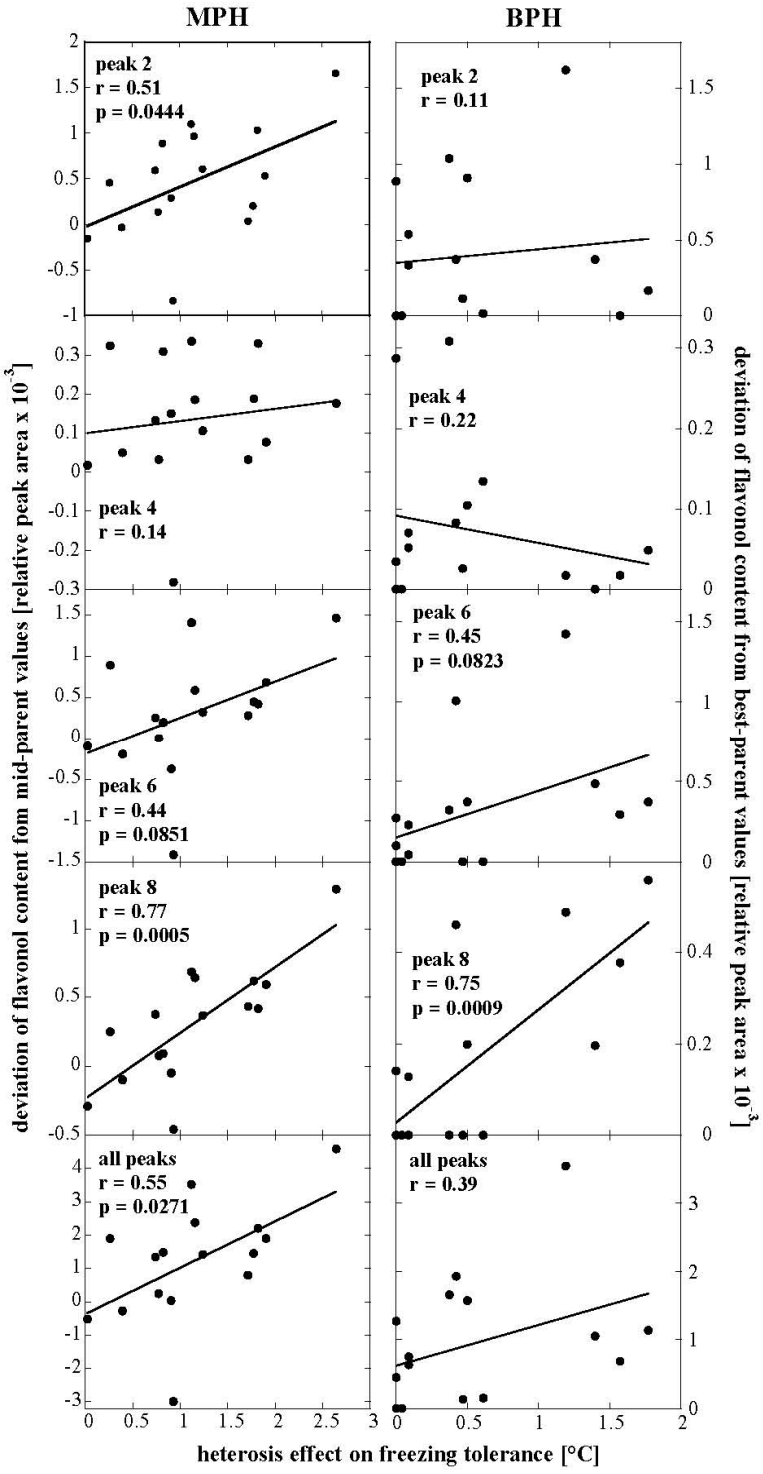

Figure 12. Analysis of the correlations between deviations from mid-parent or best-parent values in the content of different flavonols in the leaves and the heterosis effects on leaf freezing tolerance (compare Figs 2 & 10). Data were compiled from all investigated accessions and their F1 for both non-acclimated and cold-acclimated plants, and include both statistically significant and non-significant deviations. The lines were fitted to the data by least square linear regression analysis, and the correlation coefficients are shown in the panels. Correlation *P* values are only shown when they were smaller than 0.1. Analyses were performed for both mid-parent heterosis (MPH, left panels) and best-parent heterosis (BPH, right panels) in freezing tolerance.

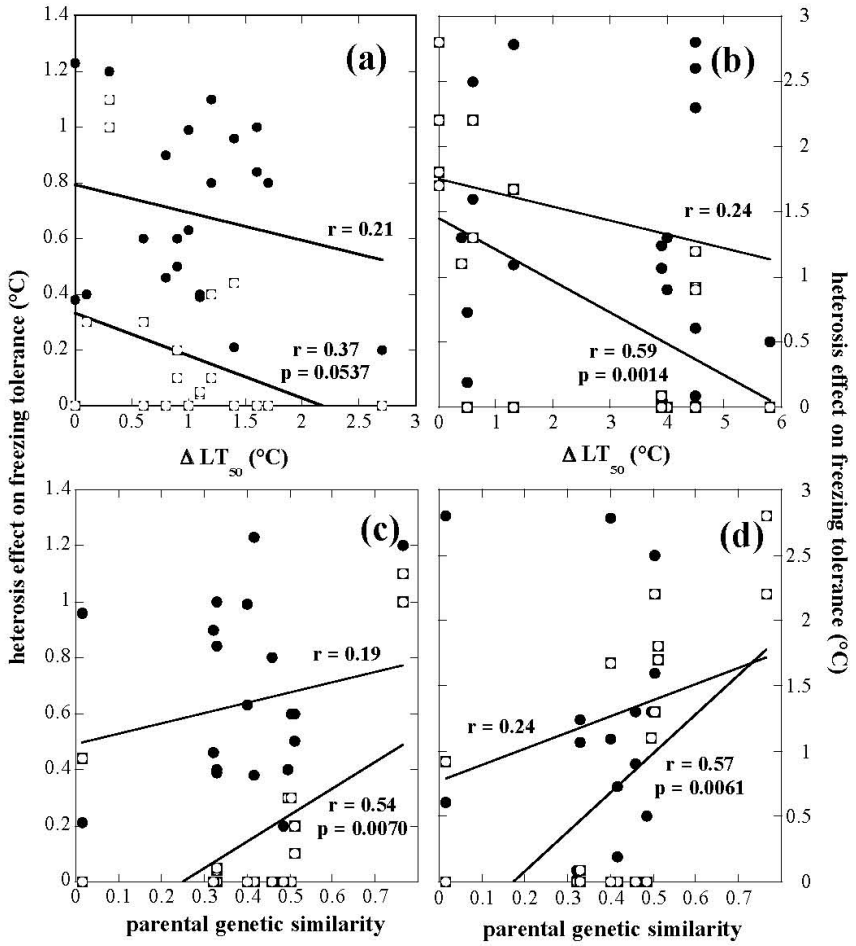

Figure 13. Dependence of the heterosis effect in leaf freezing tolerance on phenotypical (DLT_{50}; difference in freezing tolerance between the parental accessions) and genotypical (parental genetic similarity) distance. Panels (a) and (b) show the data for phenotypical distances, and panels (c) and (d) the data for genotypical differences for non-acclimated (a, c) and cold-acclimated (b, d) plants. The solid symbols represent mid-parent heterosis (MPH) and the open symbols best-parent heterosis (BPH). All lines were fitted to the data by least square linear regression analysis, and the correlation coefficients are shown next to the lines. Correlation *P* values are only shown when they were smaller than 0.1.

3.5. Discussion

While heterosis in *Arabidopsis* freezing tolerance has been reported previously (Rohde *et al.* 2004) for crosses between C24 and Col, the present study extends this to 24 crosses representing reciprocal pairs of 12 combinations of accessions.

The data show that C24 has a better combining ability than Col, as heterosis was much more frequent in crosses involving C24 than in those involving Col. Significantly, two of the three hybrids that showed BPH in crosses with Col were Col x C24 and C24 x Col described by Rohde *et al.*(2004). It is also interesting to note that heterosis in biomass production and

heterosis in freezing tolerance were not genetically related. There are five crosses that have been investigated both for freezing tolerance in our study and for biomass production (Meyer *et al.* 2004), and the magnitudes of heterosis shown by these crosses differed strongly for the two traits. Clearly, it is not general 'vigour' that determines heterosis for these two traits, but very specific combinations of alleles in the F1 hybrids. The fact that in almost all cases heterosis decreased in the F2 plants compared to the F1 is in agreement with this hypothesis.

It would be of considerable practical and theoretical interest to be able to predict the magnitude of the heterosis effect from characteristics of the parental plants. Such characteristics may be, for example, the phenotypic or genotypic similarity between parental lines. For phenotypic similarity, we found that the difference in freezing tolerance between the parental accessions and the magnitude of the heterosis effect in freezing tolerance of the respective F1 plants are in all four cases (MPH/NA, MPH/ACC, BPH/NA, BPH/ACC) negatively correlated (Fig. 13a,b). This is particularly pronounced for BPH in cold-acclimated plants (r = 0.59; P = 0.0014). It should also be mentioned at this point that the freezing tolerance of the F1 plants never exceeded that of the most freezing-tolerant parent in our study, Te. In fact, all crosses involving the two most freezing-tolerant accessions (Rsch and Te; Hannah *et al.* 2006) showed either no or very low heterosis. This could be because of the fact that these two accessions have particularly poor combining ability. However, crosses of C24 with Rsch and Te showed significant heterosis for biomass production (Meyer *et al.* 2004), making this explanation unlikely. Alternatively, there may be genetical constraints limiting freezing tolerance in *Arabidopsis*, that cannot be transgressed by crossing different accessions. The fact that the F2 populations showed reduced average heterosis compared to the F1 also argues against substantial transgression towards increased freezing tolerance.

To estimate parental genetic similarity, we used a distance matrix based on a pairwise comparison of genotypic data from 115 single nucleotide polymorphism (SNP)-based markers (Schmid *et al.* 2006). An analysis of the correlation between parental genetic similarity and the magnitude of the heterosis effects showed that in general heterosis was bigger in the genetically more similar combinations. The correlations were particularly strong for BPH in both acclimated and non-acclimated plants. This is again different from the situation for heterosis for biomass production, where a weak inverse correlation was found (Meyer *et al.* 2004), emphasizing again the basic genetical difference between heterosis in biomass production and freezing tolerance.

Metabolite profiling using GC–MS showed that the majority of detected metabolites was increased in content during cold acclimation (see Guy *et al.* 2008 for review). However, analysis of the freezing tolerance and metabolite content of nine different *Arabidopsis* accessions revealed a number of metabolites, particularly sugars, that were significantly correlated with acclimated freezing tolerance (Hannah *et al.* 2006). Here, we have analyzed

the sugar and proline content of 32 different genotypes (24 crosses and eight parental accessions) before and after cold acclimation. The content of all four sugars (Glc, Fru, Suc, Raf) showed linear correlations with freezing tolerance. Interestingly, however, the sum of all sugars showed a similar correlation. This constitutes novel evidence that the sugars may not have very specific effects, but rather constitute a redundant system of cellular stabilization, or substrates for the synthesis of other cryoprotectants, as hypothesized earlier (Klotke *et al.* 2004). The observed lack of specificity of sugars is in accord with a report that *Arabidopsis* plants that are not able to synthesize Raf, because of a knock-out (k.o.) mutation in the raffinose synthase gene show the same freezing tolerance and cold acclimation behaviour as the wild-type Col plants (Zuther *et al.* 2004), indicating that Raf does not play an indispensable role in *Arabidopsis* freezing tolerance and cold acclimation. Unfortunately, similar studies are not possible with the other three sugars, as they play crucial roles in central metabolism. Clearly, correlations represent no proof for any causality. However, it has been shown earlier that accessions that acclimate poorly nevertheless accumulate large amounts of starch over a 14 d acclimation period (Hannah *et al.* 2006; Guy *et al.* 2008), indicating that it is not the lack of available fixed carbon that limits freezing tolerance and metabolite accumulation. The data presented here on a much wider range of genotypes provide further evidence for a functional role of sugars (if not a particular sugar) in plant freezing tolerance.

A similar picture as seen for freezing tolerance emerged also from our analysis of the involvement of sugars in the heterotic effects observed in freezing tolerance. All four sugars showed MPD and BPD of their contents, especially after cold acclimation, and these were clearly correlated with heterosis in freezing tolerance. The sum of the deviations from mid-parent or best-parent values in the content of the four sugars was correlated to a similar degree with heterosis in freezing tolerance, again indicating redundancy of the different sugars.

Interestingly, the amino acid proline, which is also generally considered a compatible solute (Yancey *et al.*1982; Somero 1992), presents a different picture. While we could observe a correlation between leaf proline content and freezing tolerance, the deviations from midparent and best-parent values were weak, and there were no correlations with heterotic effects in freezing tolerance. This finding clearly shows that the correlations observed for the sugars are not just unspecific effects on all solutes, but that some solutes contribute significantly to the observed heterosis in freezing tolerance, while others do not. This is important evidence to suggest that metabolites, or specific combinations of metabolites, might be identified, which contribute significantly to the observed heterosis in freezing tolerance. A comprehensive metabolite profiling is currently underway to establish the role of the metabolome in the heterosis of *Arabidopsis* freezing tolerance. The data presented here strongly suggest that not all metabolites that accumulate during cold acclimation play a role

in the heterosis in freezing tolerance, and therefore, this is a unique experimental system to distinguish the functional roles of different metabolites or groups of metabolites.

Table 1. Identification of the flavonoids present in the peaks shown in the high-performance liquid chromatography (HPLC) elution profiles in Fig. 9

			Compound masses (M + H)+		
Peak number	Compound class	UV/Vis lmax (nm)	ESI–MS (*m*/*z*)	MS fragments (*m*/*z*)	Predicted compounds
1	Flavonol derivative	256/356	757	454	Quercetin–Rha–Rha–Glc
2	Flavonol derivative	266/348	741	496	Not identified
3	Anthocyanin	527	Not determined (only detectable in Te)		Not identified
4	Flavonol derivative	266/347	757	611	Quercetin–Rha–Rha–Glc
5	Flavonol derivative	266/345	757	595/432	Kaempferol–Glc–Glc–Rha
6	Flavonol derivative	266/347	595	476	Kaempferol–Rha–Glc
7	Anthocyanin	544	Not determined (only detectable in Te)		
8	Flavonol derivative	265/345	579	433	Kaempferol–Rha–Rha
9	Cinnamic acid derivative	331	363		Sinapoyl malate
10	Cinnamic acid derivative	317			Not identified

Compounds were identified by photodiode array detector (PDA) absorbance spectra and mass spectrometry. Rha, rhamnose; Glc, glucose.

The flavonoid biosynthesis pathway in *Arabidopsis* is regulated by the MYB family transcription factors PAP1 and PAP2, which control the regulon of genes encoding the flavonoid biosynthesis pathway enzymes (Tohge *et al.*2005). The *PAP1* gene is up-regulated by Suc (Teng *et al.* 2005), while *PAP2* is strongly cold induced at the transcript level (Hannah *et al*. 2005, 2006) indicating that although Suc is accumulated in cold-acclimated plants, the induction of flavonoid biosynthesis during cold acclimation follows a separate signalling pathway. The expression of *PAP2* is strongly correlated with the acclimated freezing tolerance of the *Arabidopsis* accessions used in the present study (Hannah *et al.* 2006). However, whether the observed differences in gene expression are reflected in the flavonoid content of leaves, and whether this is also correlated with leaf freezing tolerance, have previously not been investigated. Comprehensive profiling of the flavonoid composition of five accessions and eight F1 hybrids, both before and after cold acclimation, showed here for the first time that there is considerable natural variation in *Arabidopsis* flavonol composition. In addition, cold acclimation has a major influence on the amount and composition of flavonols, in agreement with the gene expression data.

The content of all major flavonols was linearly correlated with leaf freezing tolerance. These correlations were in general stronger than those between sugars and freezing tolerance

(r = 0.83–0.90 versus r = 0.66–0.70), indicating that flavonols may indeed play a functional role in plant freezing tolerance. A possible caveat in this comparison is the difference in sample size, because we investigated fewer genotypes for flavonoid than for sugar content. However, when we performed the correlation analyses for the sugar content data with only those genotypes that were also investigated for flavonoid content, we found almost unchanged r values, but because of the smaller sample size, higher P values (0.0293–0.0824; data not shown). This strongly supports our conclusions and emphasizes the high significance of the correlations observed for the flavonols. This is the first evidence that flavonols may play a functional role in plant cold acclimation and freezing tolerance.

In addition, all major flavonols showed significant deviations from mid-parent and best-parent values in several crosses, especially after cold acclimation. The degree of correlation between MPD and BPD in flavonol content and heterosis in freezing tolerance was much more variable between the different flavonols than between different sugars, indicating that some of the flavonols may play more specific roles in freezing tolerance than the sugars. Especially striking is the fact that both MPD and BPD in the content of the flavonol in peak 8 are much more strongly correlated (higher r and lower P values) with heterosis in freezing tolerance than the respective values for the content of the flavonol in peak 6, although both peaks contain kaempferol glycosides. The functional basis of this difference is presently unclear, but our results strongly indicate this to be an interesting area of further research.

Several stress-related functions in plants have been proposed for flavonoids in the literature, for example, protection against UV-B radiation and antioxidant activity (see Rice-Evans,Miller & Paganga 1997; Harborne &Williams 2000; Winkel-Shirley 2002 for comprehensive reviews). However, UV-B-absorbing flavonoids are located in the upper epidermal cell layer and in the cuticular wax layer, and would not contribute to the freezing tolerance of other cells in a leaf. In addition, our freezing experiments are performed in the dark, and cold acclimation is performed in climate chambers with artificial lighting that does not produce significant amounts of UV-B radiation. Therefore, protection against UV-B radiation can be excluded as a mechanism contributing to freezing tolerance and cold acclimation in our experiments.

However, cold-induced flavonoids have been shown to be effective UV-B screens in a number of different species (Bilger, Rolland & Nybakken 2007), indicating that under natural conditions, this may be an important part of the adaptation processes of cold acclimation.

The scavenging of reactive oxygen species (ROS) during cold acclimation and during freezing may be another protective mechanism afforded by flavonoids, and it has been shown that flavonols such as quercetin are especially potent antioxidants (Rice-Evans *et al.* 1997). Further experiments will be necessary to evaluate the contribution of ROS to freezing damage under our experimental conditions and possible scavenging effects of different flavonols, for

example, in the chloroplasts (Agati *et al.* 2007). In addition to ROS scavenging, flavonols may also have direct effects on the stability of cellular membranes. It has been shown that flavonols can partition into the lipid phase of membranes (Scheidt *et al.* 2004). Under freezing conditions, when a large part of the water is removed from the cells to intercellular ice crystals, it can be expected that amphiphiles such as flavonoids will partition even more strongly into the hydrophobic phase of membranes (Hoekstra & Golovina 2002).While this can be disruptive in some cases (Ollila *et al.* 2002; Tamba *et al.* 2007), it has also been shown for a glucosylated phenol (arbutin; 4-hydroxyphenol-b-d-glucopyranoside) that it is able to specifically stabilize membranes that contain non-bilayer lipids (Hincha, Oliver & Crowe 1999, Oliver *et al.* 2001). Similar mechanisms could be envisaged also for flavonoids, but this hypothesis needs to be experimentally tested in the future.

3.6. Acknowledgments

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3.8. Supplementary material

The following supplementary material is available for this article:

Table S1. Statistical significance of deviations from midparent or best-parent values (MPD, mid-parent deviation; BPD, best-parent deviation) in the sugar content of crosses between *Arabidopsis* accessions, indicated as *P* values from *t*-tests. Plants were harvested after growth under nonacclimating conditions (NA), or after an additional 14 d of cold acclimation at 4 °C (ACC). Values in bold indicate *P* values <0.05. n.a., not applicable (significant negative BPD); n.d., not determined.

		Glucose		Fructose		Sucrose		Raffinose	
	Accession	MPD	BPD	MPD	BPD	MPD	BPD	MPD	BPD
NA	C24 x Can	**<0.0001**	**<0.0001**	**<0.0001**	**0.0013**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
ACC	C24 x Can	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.3995	0.1193
NA	Can x C24	**<0.0001**	**<0.0001**	**<0.0001**	**0.0002**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
ACC	Can x C24	**<0.0001**	**<0.0001**	**<0.0001**	**0.0019**	**<0.0001**	**0.0004**	0.797	n.a.
NA	C24 x Co	**0.0135**	0.0744	**0.0003**	**0.0105**	0.0505	0.4035	**0.0006**	**0.0211**
ACC	C24 x Co	**<0.0001**	**<0.0001**	**<0.0001**	**0.0002**	**<0.0001**	**0.0069**	**<0.0001**	**<0.0001**
NA	Co x C24	0.3848	0.5374	**0.0376**	0.169	0.0788	0.3696	**0.0022**	**0.0418**
ACC	Co x C24	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0018**	**<0.0001**	**0.0007**
NA	C24 x Cvi	0.2696	0.5587	0.164	0.3318	0.1529	**0.0432**	**0.0075**	**0.0736**
ACC	C24 x Cvi	**<0.0001**	**<0.0001**	**<0.0001**	**0.0042**	**<0.0001**	**0.0007**	**0.0013**	0.1261
NA	Cvi x C24	**0.0004**	**0.0136**	**0.0007**	**0.0145**	**0.0058**	**0.0076**	**0.0002**	**0.0114**
ACC	Cvi x C24	**<0.0001**	**0.0005**	**<0.0001**	**0.0025**	**<0.0001**	**0.0015**	**<0.0001**	**0.0192**
NA	C24 x Ler	0.7557	0.3388	0.5727	0.6664	**0.0004**	0.8483	0.5389	0.3245
ACC	C24 x Ler	**0.0017**	0.1118	**0.0063**	0.1693	**0.0175**	0.094	**0.0212**	0.1361
NA	Ler x C24	**0.0212**	0.571	0.9904	0.1295	0.1483	0.0736	**0.0465**	0.1209
ACC	Ler x C24	**0.0002**	0.0558	**0.0009**	0.0956	**<0.0001**	**0.0114**	**0.0102**	0.0957
NA	C24 x Rsch	**0.0118**	0.0911	**0.0073**	0.0826	0.804	n.a.	**0.002**	0.0583
ACC	C24 x Rsch	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0134**	0.0883	**0.0006**	0.0939
NA	Rsch x C24	**0.0002**	**0.0087**	**0.0003**	**0.014**	0.9804	0.0891	0.0725	0.4782
ACC	Rsch x C24	**<0.0001**	**0.0013**	**<0.0001**	**0.0217**	0.0694	0.1972	0.2124	0.803
NA	C24 x Ten	**<0.0001**	**<0.0001**	**0.0006**	**0.0351**	**<0.0001**	**0.0051**	**<0.0001**	**<0.0001**
ACC	C24 x Ten	**0.0295**	0.2936	**<0.0001**	**0.0166**	0.093	0.0996	**0.0009**	0.8757
NA	Ten x C24	**<0.0001**	**0.0039**	**0.0201**	0.2599	**0.0053**	0.1161	**<0.0001**	**<0.0001**
ACC	Ten x C24	**<0.0001**	**0.0104**	**<0.0001**	**0.0043**	**0.0026**	0.0575	**<0.0001**	**0.0469**
NA	Col x Can	**0.0024**	**0.0396**	0.0788	0.4991	0.692	0.0874	**0.0071**	0.1222
ACC	Col x Can	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
NA	Can x Col	0.2623	0.8501	0.4408	0.9248	0.1753	n.a.	0.7435	0.0759
ACC	Can x Col	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
NA	Col x Co	0.4287	0.7045	0.4361	0.2803	0.4056	0.7677	0.4954	0.1867
ACC	Col x Co	0.1641	0.4185	0.1972	0.3388	0.453	0.1628	0.2498	0.1878
NA	Co x Col	0.1881	0.2898	0.5177	0.945	0.8505	0.8478	0.3768	0.8359
ACC	Co x Col	**0.0002**	**0.0013**	**0.0012**	**0.0044**	**0.0215**	0.0645	0.1873	0.4919
NA	Col x Cvi	0.0963	0.7202	0.301	0.2715	0.4759	0.0547	0.8242	0.8072
ACC	Col x Cvi	0.1602	n.a.	**0.0472**	n.a.	0.0928	0.0601	**0.0355**	n.a.
NA	Cvi x Col	**0.0053**	**0.0009**	0.123	0.1291	**0.0016**	n.a.	**0.0665**	n.a.
ACC	Cvi x Col	0.2778	0.5362	0.5885	0.728	0.6134	0.3177	0.0571	n.a.
NA	Col x Ler	0.1421	0.1887	0.6332	0.8127	**0.0004**	**0.0071**	0.2017	0.3564
ACC	Col x Ler	0.5043	0.0679	0.8155	0.6031	0.5842	0.6872	0.7976	0.3655
NA	Ler x Col	**0.0052**	**0.0155**	0.9464	0.8354	**0.0025**	0.0519	0.4365	0.6377
ACC	Ler x Col	**0.0054**	n.a.	**0.0276**	**0.0093**	**0.025**	n.a.	0.1247	n.a.
NA	Col x Rsch	0.2776	0.5349	**0.0481**	n.a.	0.4286	0.2879	0.4085	0.1741
ACC	Col x Rsch	0.2002	0.6571	0.2884	0.4122	0.1881	0.5049	0.1469	0.6231
NA	Rsch x Col	0.7378	0.6775	**0.0402**	n.a.	0.6545	0.4286	0.107	0.0607
ACC	Rsch x Col	**0.0257**	0.1191	**0.0183**	0.2994	**0.0311**	0.0733	0.0828	0.3728
NA	Col x Ten	0.6868	0.0652	0.3517	n.a.	0.0747	0.719	0.2193	n.a.
ACC	Col x Ten	0.0629	0.8183	**0.0173**	0.3805	0.6647	n.a.	0.101	0.3719
NA	Ten x Col	0.1319	n.a.	0.094	n.a.	0.5427	n.a.	0.1693	n.a.
ACC	Ten x Col	0.1482	0.8247	0.0966	0.4325	0.4871	0.1453	0.6855	0.7761

Table S2. Statistical significance of deviations from midparent or best-parent values (MPD, mid-parent deviation; BPD, best-parent deviation) in the flavonol content of crosses between *Arabidopsis* accessions, indicated as *P* values from *t*-tests. Plants were harvested after growth under non-acclimating conditions (NA), or after an additional 14 d of cold acclimation at 4 °C (ACC). The peak numbering refers to the high-performance liquid chromatography (HPLC) elution profile shown in Fig. 9. Further information about the analyzed compounds can be found in Table 1. Values in bold indicate *P* values <0.05. n.a., not applicable [significant negative best-parent deviation (BPD)].

		Peak2		Peak4		Peak6		Peak8	
	Accession	MPD	BPD	MPD	BPD	MPD	BPD	MPD	BPD
NA	C24 x Co	0.7486	0.2033	0.2313	0.5384	**0.0005**	**0.002**	**0.0003**	**0.0022**
ACC		0.3469	0.4435	0.1693	0.7356	0.1851	0.2577	**0.0006**	**0.0003**
NA	Col x Co	**0.0014**	**0.0317**	**0.0159**	0.5881	**0.0007**	**0.0048**	**0.0017**	0.1995
ACC		**<0.0001**	**<0.0001**	0.0805	0.8832	**<0.0001**	**<0.0001**	**0.0002**	0.064
NA	C24 x Col	**<0.0001**	**<0.0001**	**<0.0001**	**0.0006**	**0.0003**	**0.0114**	**0.0002**	0.1663
ACC		**<0.0001**	**<0.0001**	**0.0005**	**0.0003**	0.27	0.4748	0.4519	n.a.
NA	Col x C24	**<0.0001**	**<0.0001**	**<0.0001**	**0.0028**	**0.0017**	0.5757	**0.002**	0.5326
ACC		**<0.0001**	**<0.0001**	**0.0007**	**0.0006**	0.0521	0.0645	**0.0004**	n.a.
NA	C24 x Ler	**0.0188**	**0.0255**	0.0913	0.1145	0.8883	n.a.	0.2659	n.a.
ACC		**0.0022**	0.882	**0.0355**	**0.0304**	**0.0411**	n.a.	0.3907	n.a.
NA	Col x Ler	0.5471	0.0526	**0.0012**	0.1544	**0.0091**	n.a.	0.3173	n.a.
ACC		**0.0005**	**0.0313**	0.0904	0.1087	**0.0273**	0.129	**0.0067**	0.3431
NA	C24 x Te	0.265	n.a.	0.7282	0.191	0.4734	n.a.	**0.0029**	n.a.
ACC		**0.0003**	n.a.	**0.0005**	n.a.	**<0.0001**	n.a.	**0.0005**	n.a.
NA	Col x Te	**<0.0001**	0.0706	**0.0002**	0.2102	**0.0002**	**0.0029**	**0.0004**	**0.0066**
ACC		**0.0068**	n.a.	**0.0112**	0.7512	**0.0016**	0.2143	**0.0127**	0.1709

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4. Manuscript 1 (M1)

Metabolic heterosis associated with heterosis in freezing tolerance of *Arabidopsis thaliana*

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Authors contribution

The research was conceived and planned by Dirk K. Hincha and Marina Korn. The experimental work was done by Marina Korn. Metabolite measurement was done in cooperation with Alexander Erban and evaluated by Marina Korn.

4.1. Abstract

Although heterosis, the enhanced performance of hybrids compared to their parents, is one of the most important genetical tools in crop plant breeding, its molecular basis is not understood. We used metabolic profiling to discover metabolites contributing to heterosis in Arabidopsis freezing tolerance and identified the TCA cycle as central for the heterotic phenotype. This indicates coordinate effects of heterosis on metabolic pathways and the existence of heterotic regulator molecules.

4.2. Results & Discussion

The term heterosis describes the phenomenon of increased physiological performance of hybrids compared to their parents, where mid-parent heterosis denotes the deviation of the F1 from the parental mean (MPH = F1 – (P1 + P2)/2). Although heterosis is one of the most important tools in crop plant breeding (1), its molecular basis is largely unknown 2.

Arabidopsis thaliana is a valuable model plant to study the genetic and molecular basis of quantitative traits through the analysis of natural variation 3. Heterosis in crosses between Arabidopsis accessions has been shown e.g. for biomass accumulation 4 and freezing tolerance 5. We recently identified five accessions and eight crosses that span the range of freezing tolerance and the corresponding heterosis described in Arabidopsis 6.

Freezing tolerance limits crop yields and the geographic distribution of plants. In Arabidopsis it is correlated with latitude of origin and habitat growth temperature 7. Cold acclimation at low nonfreezing temperatures enables many plants to increase their freezing tolerance 8. This is a multigenic, quantitative trait and in Arabidopsis, transcript and metabolite profiling revealed massive reprogramming of gene expression and metabolism 9.

We have profiled primary metabolites (Supplemental Table 1) by gas chromatography mass spectrometry (GC-MS) in five parental accessions (C24, Col-0, Co-2, L*er*, Te) and eight F1 populations generated by crossing both C24 and Col-0 with the other four accessions 6. In three independent experiments, five replicate plants of each genotype were harvested either before or after 14 days of cold acclimation at 4°C 6. Methods for the extraction of polar metabolites, GC-MS measurements and metabolite identification and quantification have been published 10,11.

Supplemental Figure 1 gives an overview of the changes in metabolite pool sizes in the parental accessions during cold acclimation. The largest changes occurred in C24 and Co-2,

the smallest in Te, with L*er* and Col-0 intermediate. This ranking was also observed previously [7], attesting to the reproducibility of these metabolic effects over several years.

Heterotic effects on metabolite pools were analyzed by comparing the mean metabolite levels of the parental accessions to the metabolite content of the respective F1 plants. Thus we followed the same strategy of monitoring relative changes in metabolite pools that led to the discovery of metabolic QTL in Arabidopsis [12] and tomato [13,14]. Figure 1 gives an overview of heterotic effects on the pool sizes of identified metabolites in all crosses. There were strongly and weakly affected metabolites and while most metabolites showed positive MPH in all crosses, some showed both positive and negative MPH, depending on the hybrid.

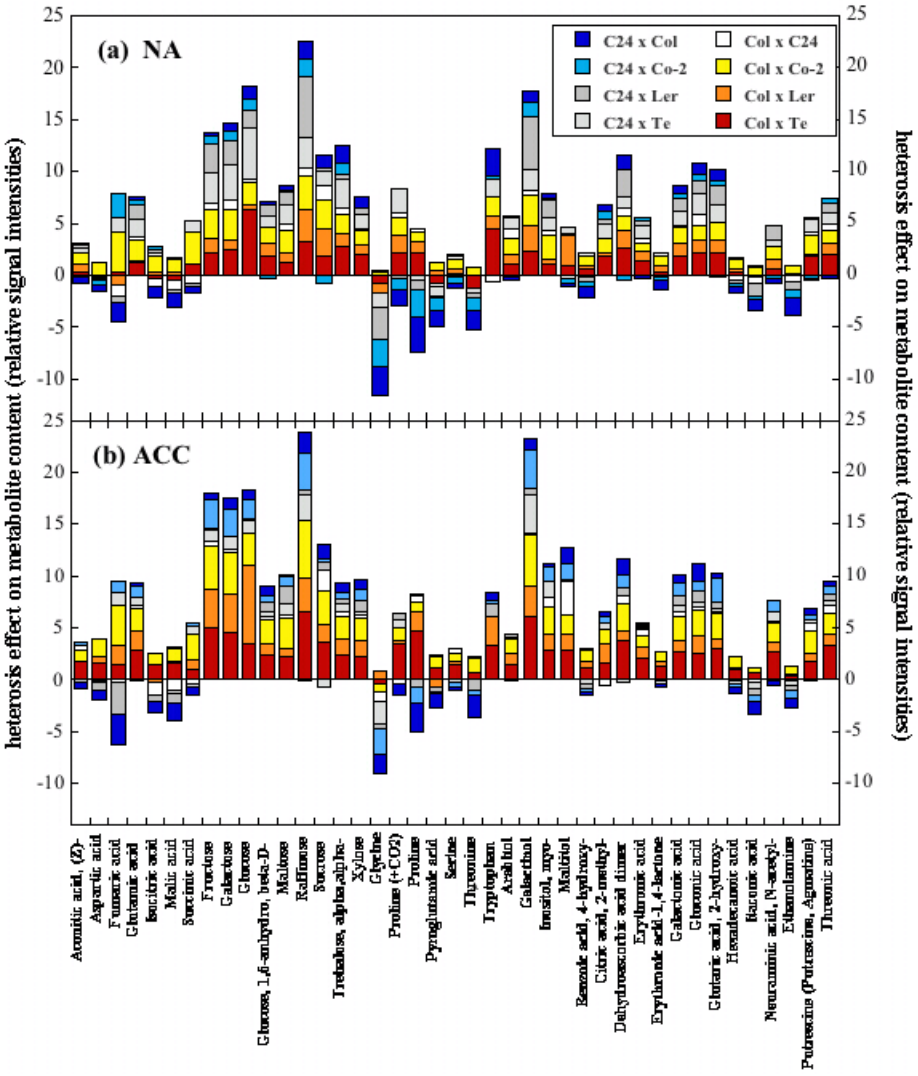

Figure 1. Mid-parent heterosis (MPH = F1 – (P1 + P2)/2) in the metabolite content of rosette tissue from eight F1 populations before (NA; a) or after (ACC; b) 14 days of cold acclimation at 4°C.

Figure 2 shows a comparison of the metabolite contents of the F1 plants (Fig. 2a) with MPH of these metabolites from the same samples (Fig. 2b). As in the parental accessions [7] there was a division between metabolite profiles of nonacclimated and acclimated plants (Fig. 2a) indicating massive metabolic changes during cold acclimation. This was no longer apparent when MPH of the metabolite pools was used for cluster analysis (Fig. 2b), indicating that the genetical impact on metabolite content was stronger than the massive metabolic remodeling at low temperature.

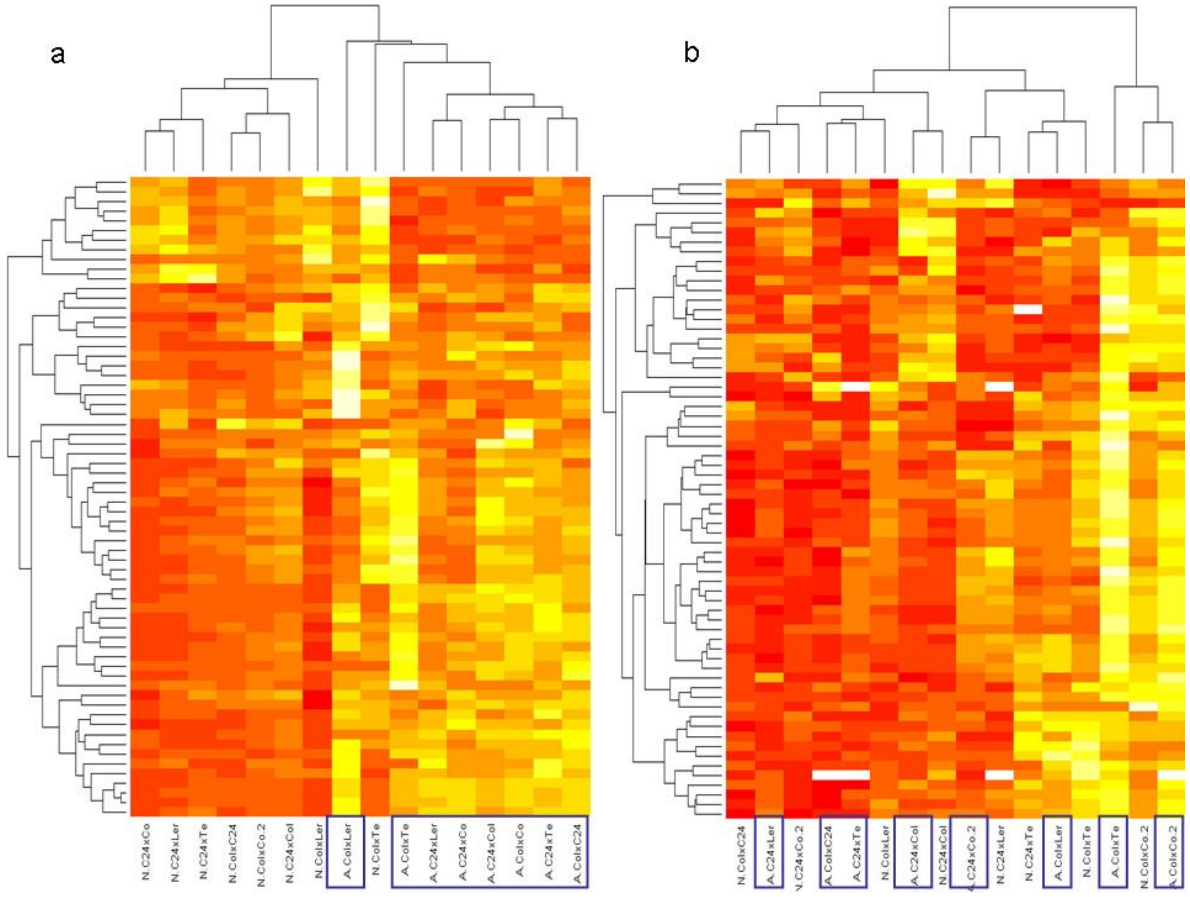

Figure 2. Hierarchical clustering of the metabolite contents in all crosses before (N) and after (A) cold acclimation. The cold acclimated samples are highlighted by blue boxes. In **Fig. 2a**, normalized peak areas were scaled to unit variance. Red indicates the lowest and yellow the highest metabolite content. In **Fig. 2b** absolute MPH in metabolite levels was scaled to unit variance and red indicates the smallest and yellow the largest MPH. In both panels, white indicates missing values and Euclidian distance was used for clustering.

To identify the contribution of specific metabolites to freezing tolerance and heterosis, we performed correlation analysis. Supplemental Table 1 shows that the content of several metabolites correlated significantly with the freezing tolerance of the parental and F1 populations. In addition, when we correlated metabolite MPH with heterosis in freezing

tolerance, an additional set of metabolites was identified that showed only little overlap with the previous sets of correlating metabolites. Strikingly, all compatible solutes often associated with plant freezing tolerance, such as sucrose and raffinose, only correlated with freezing tolerance, but were not in the list of correlating heterotic metabolites. This indicates that at the metabolic level quantitative differences in the trait itself and in heterotic effects in the trait may not be closely linked, indicating an independent genetical basis for natural variation in freezing tolerance and for the magnitude of heterotic effects, in agreement with the finding that heterosis is not correlated with differences in freezing tolerance between the parental accessions [6].

Interestingly, five out of the eight metabolites that had a correlation at $p \leq 0.05$ between heterosis of pool size and heterosis in freezing tolerance belong to a central metabolic pathway, the tricarboxylic acid (TCA) cycle (Fig. 3). The coordinate involvement of a complete metabolic pathway suggested by these correlations may indicate that heterosis could be related to effects on regulatory genetical elements, which might be identified as distinct loci through heterotic QTL mapping. Increased amounts of some TCA cycle intermediates during cold acclimation have been reported previously [9]. Moreover, one crucial enzyme of the

TCA cycle (⟨-ketoglutarate dehydrogenase), was found to be highly sensitive to oxidative stress leading to a block in this metabolic pathway [15]. In the case of heterosis, all significant correlations were negative, indicating that larger MPH in freezing tolerance may be related to a smaller pool size of TCA cycle intermediates in the F1 as compared to the parental mean. Functionally, this could indicate either restricted flux into or higher flux from the TCA cycle for respiratory energy production or for biosynthetic processes coupled to TCA cycle activity. This has to be resolved by flux analysis and feeding of isotopically labeled precursors.

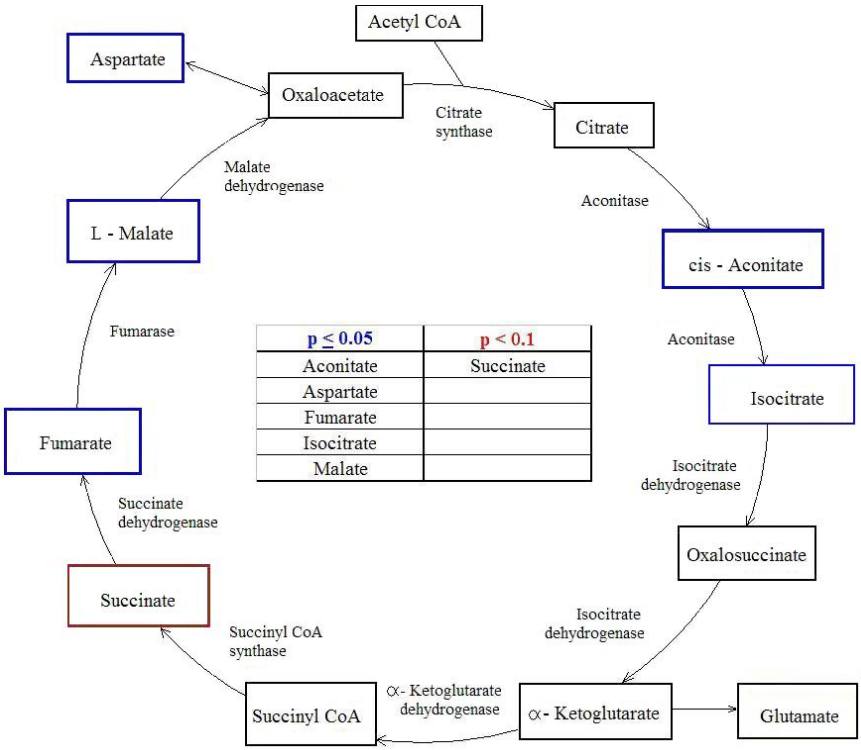

Figure 3. Schematic representation of the TCA cycle. Metabolite names are shown in the cycle and the names of the relevant enzymes inside the cycle. Metabolites boxed in blue showed a p-value below 0.05 and those boxed in red showed a p-value below 0.1 in the analysis of correlations between metabolite MPH and MPH in freezing tolerance (Supplemental Table 1). Metabolites boxed in black were not measured and the correlation for glutamic acid was not significant.

4.3. Acknowledgments

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4.4. References

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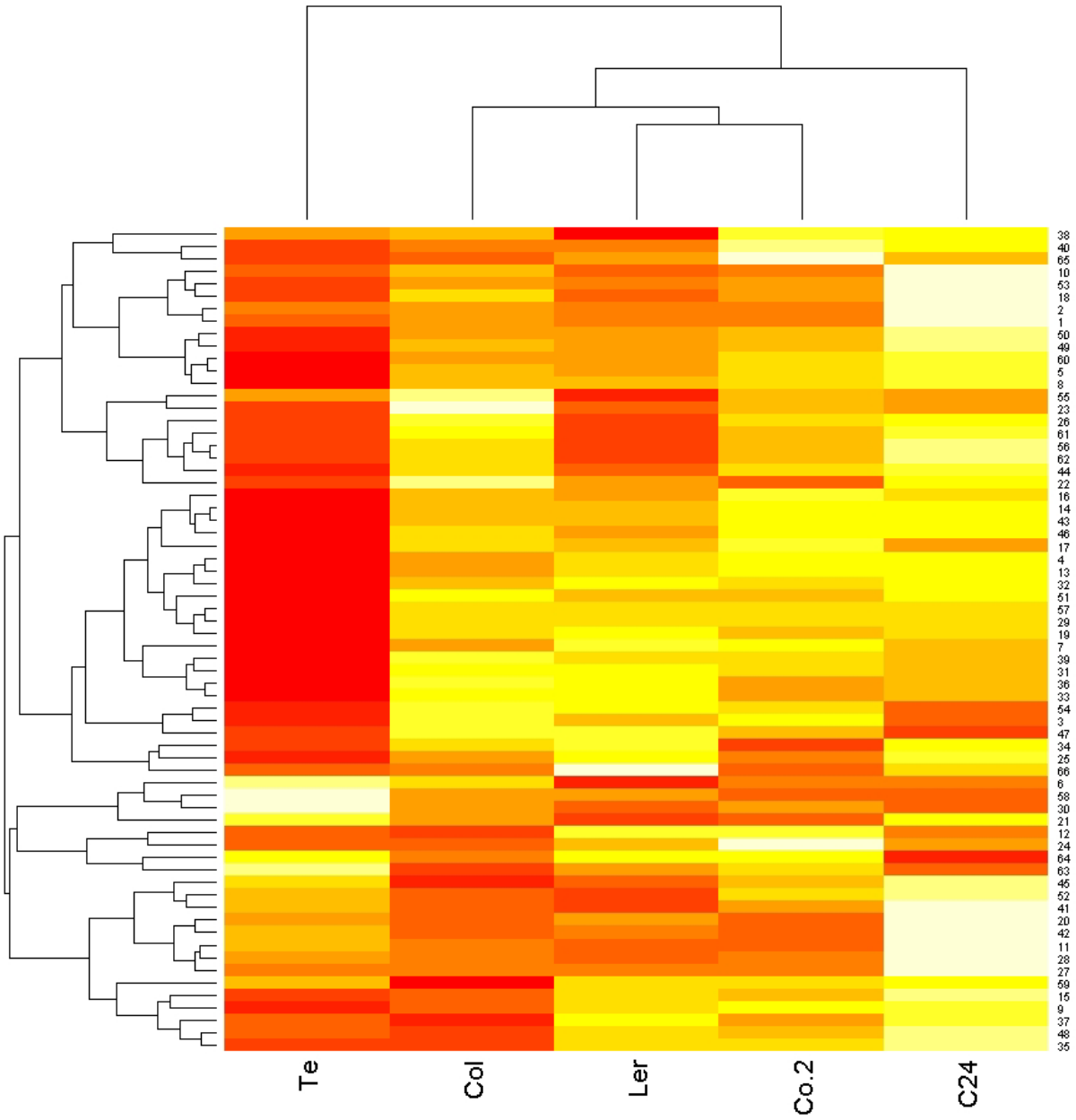
4.5. Supplemental Material

Supplemental Table 1. A list of all analyzed metabolites, identified by common name, MPIMP-ID from the Golm Metabolome Database and CAS-ID for the analytes, sum formula, KEGG-ID and CAS-ID for the metabolites. In addition, the results from correlation analyses between metabolite content and freezing tolerance (LT_{50}), and between metabolite MPH and freezing tolerance MPH are listed.

A		**B**				**Metabolite name**	**Analyte**		**Metabolite**		
all crosses		**C24 - crosses**		**Col - crosses**							
R	**p**	**R**	**p**	**R**	**p**		**MPIMP-ID**	**CAS- ID**	**Sum Formula**	**KEGG-ID**	**CAS-ID**
-0.5732	**0.019**	-0.123	n.s.	0.219	n.s.	**Aconitic acid, cis-**	A176002-101	55530-71-7	C6H6O6	C00417	585-84-2
-0.6673	**0.008**	0.557	**0.013**	0.573	**0.035**	**Aspartic acid**	A144003-101	N.A.	C4H7NO4	C00049	56-84-8
-0.6124	**0.01**	0.311	n.s.	0.487	*0.089*	**Fumaric acid**	A137001-101	17962-03-7	C4H4O4	C00122	110-17-8
-0.2567	n.s.	-0.609	**0.003**	-0.748	**0.002**	**Glutamic acid**	A163001-101	15985-07-6	C5H9NO4	C00025	56-86-0
-0.4748	**0.05**	-0.104	n.s.	0.087	n.s.	**Isocitric acid**	A182003-101	N.A.	C6H8O7	C00311	320-77-4
-0.5224	**0.034**	-0.425	*0.074*	-0.338	n.s.	**Malic acid**	A149001-101	38166-11-9	C4H6O5	C00149	97-67-6
-0.4533	*0.075*	0.444	**0.050**	0.718	**0.002**	**Succinic acid**	A134001-101	40309-57-7	C4H6O4	C00042	110-15-6
-0.2156	n.s.	-0.685	**0.000**	-0.760	**0.002**	Fructose	A187002-101	56196-14-6	C6H12O6	C00095	57-48-7
-0.1749	n.s.	-0.718	**0.000**	-0.830	**0.000**	Galactose	A191002-101	N.A.	C6H12O6	C00124	N.A.
0.0970	n.s.	-0.584	**0.007**	-0.812	**0.000**	Glucose	A189002-101	34152-44-8	C6H12O6	C00031	50-99-7
-0.2364	n.s.	-0.511	**0.021**	-0.752	**0.003**	Glucose, 1,6-anhydro	A172001-101	7449-14-1	C6H10O5	N.A.	498-07-7
-0.2759	n.s.	-0.463	**0.042**	-0.629	**0.020**	Maltose	A274001-101	N.A.	C12H22O11	C00897	69-79-4
-0.2688	n.s.	-0.725	**0.000**	-0.816	**0.001**	Raffinose	A337002-101	N.A.	C18H32O16	C00492	512-69-6
-0.2304	n.s.	-0.502	**0.025**	-0.577	**0.036**	Sucrose	A264001-101	19159-25-2	C12H22O11	C00089	57-50-1
-0.0924	n.s.	-0.521	**0.021**	-0.561	*0.052*	Trehalose, alpha,alpha-	A274002-101	N.A.	C12H22O11	C01083	99-20-7
0.0148	n.s.	-0.612	**0.004**	-0.828	**0.000**	Xylose	A166001-101	N.A.	C5H10O5	C00181	58-86-6
-0.3058	n.s.	-0.214	n.s.	-0.297	n.s.	Arabitol	A171012-101	14199-73-6	CH5H12O5	C00532	488-82-4
-0.3131	n.s.	-0.652	**0.002**	-0.781	**0.001**	Galactinol	A299002-101	N.A.	C12H22O11	C01235	N.A.
-0.4725	*0.051*	-0.545	**0.010**	-0.719	**0.004**	Inositol, myo-	A209002-101	2582-79-8	C6H12O6	C00137	87-89-8
-0.1881	n.s.	-0.387	*0.098*	-0.629	**0.021**	Maltitol	A284001-101	N.A.	C12H24O11	N.A.	585-88-6
-0.0609	n.s.	-0.017	n.s.	0.218	n.s.	Glycine	A133001-101	5630-82-0	C2H5NO2	C00037	56-40-6
-0.1628	n.s.	-0.383	n.s.	-0.258	n.s.	Proline [+CO_2]	A159001-101	N.A.	C5H9NO2	N.A.	N.A.
-0.3241	n.s.	-0.317	n.s.	-0.311	n.s.	Proline*	A117006-101	N.A.	C5H9NO2	C00148	147-85-3
-0.6024	**0.015**	0.053	n.s.	0.147	n.s.	Pyroglutamic acid (Glutamine, Glutamic acid)	A153002-101	213608-51-6	C5H7NO3	C02238	N.A.
-0.3757	n.s.	-0.503	**0.020**	-0.399	n.s.	Serine	A128001-101	70125-39-2	C3H7NO3	C00065	56-45-1
-0.3980	n.s.	0.087	n.s.	0.104	n.s.	Threonine	A140001-101	64569-35-3	C4H9NO3	C00188	72-19-5
-0.0672	n.s.	-0.581	**0.008**	-0.565	*0.055*	Tryptophan	A223001-101	55429-28-2	C11H12N2O2	C00078	73-22-3
-0.3735	n.s.	0.224	n.s.	0.614	**0.023**	Benzoic acid, 4-hydroxy-	A164003-101	2078-13-9	C7H6O3	C00156	99-96-7
-0.0464	n.s.	-0.311	n.s.	-0.536	**0.048**	Citric acid, 2-methyl-	A185008-101	N.A.	C7H10O7	C02225	N.A.
-0.1558	n.s.	-0.677	**0.001**	-0.842	**0.000**	Dehydroascorbic acid dimer	A185002-101	N.A.	C6H6O6	C00425	490-83-5

-0.2315	n.s.	-0.271	n.s.	-0.451	n.s.	Erythronic acid	A154001-101	N.A.	C4H8O5	N.A	15667-21-7
-0.3590	n.s.	0.107	n.s.	0.590	**0.027**	Erythronic acid-1,4-lactone	A144008-101	55220-75-2	N.A.	N.A.	N.A.
-0.2747	n.s.	-0.578	**0.007**	-0.813	**0.002**	Galactonic acid	A199002-101	55400-16-3	C6H12O7	C00880	576-36-3
0.0366	n.s.	-0.407	*0.076*	-0.697	**0.008**	Gluconic acid	A200001-101	34290-52-3	C6H12O7	C00257	526-95-4
-0.1993	n.s.	-0.507	**0.019**	-0.649	**0.011**	Glutaric acid, 2-hydroxy-	A158010-101	55530-62-6	C5H8O5	C02630	2889-31-8
-0.4434	*0.066*	0.344	n.s.	0.788	**0.001**	Hexadecanoic acid	A205001-101	N.A.	C16H32O2	C00249	57-10-3
-0.3718	n.s.	-0.462	**0.044**	-0.712	**0.008**	Threonic acid	A156001-101	38191-88-7	C4H8O5	C01620	7306-96-9
-0.4755	*0.053*	0.520	**0.019**	0.794	**0.000**	Itaconic acid	A135004-101	55494-04-7	C5H6O4	C00490	97-65-4
-0.4748	*0.051*	0.525	**0.018**	0.808	**0.000**	Ethanolamine	A128002-101	5630-81-9	C2H7NO	C00189	N.A.
-0.4043	n.s.	-0.321	n.s.	-0.540	*0.052*	Neuraminic acid, N-acetyl-	A263005-101	N.A.	C11H19NO9	C00270	131-48-6
-0.3752	n.s.	-0.379	n.s.	-0.644	**0.020**	Putrescine (Putrescine, Agmatine)	A175002-101	39772-63-9	C4H12N2	C00134	110-60-1
-0.5277	**0.03**	0.295	n.s.	0.601	**0.027**	N.A.	A144006-101	N.A.	N.A.	N.A.	N.A.
-0.4750	*0.055*	0.178	n.s.	0.478	*0.085*	N.A.	A147001-101	N.A.	N.A.	N.A.	N.A.
-0.4370	*0.072*	-0.111	n.s.	0.165	n.s.	N.A.	A159003-101	N.A.	N.A.	N.A.	N.A.
-0.3002	n.s.	-0.584	**0.003**	-0.779	**0.002**	N.A.	A170002-101	N.A.	N.A.	N.A.	N.A.
-0.3060	n.s.	-0.616	**0.004**	-0.787	**0.003**	N.A.	A176001-101	N.A.	N.A.	N.A.	N.A.
0.2047	n.s.	-0.705	**0.000**	-0.808	**0.000**	N.A.	A196004-101	N.A.	N.A.	N.A.	N.A.
-0.1448	n.s.	-0.423	*0.066*	-0.654	**0.011**	N.A.	A211001-101	N.A.	N.A.	N.A.	N.A.
-0.4831	**0.042**	-0.679	**0.001**	-0.845	**0.000**	N.A.	A213001-101	N.A.	N.A.	N.A.	N.A.
-0.4136	n.s.	-0.660	**0.002**	-0.824	**0.000**	N.A.	A214004-101	N.A.	N.A.	N.A.	N.A.
-0.3380	n.s.	-0.614	**0.001**	-0.786	**0.002**	N.A.	A217003-101	N.A.	N.A.	N.A.	N.A.
-0.3934	n.s.	-0.018	n.s.	0.299	n.s.	N.A.	A227001-101	N.A.	N.A.	N.A.	N.A.
-0.0001	n.s.	-0.485	**0.020**	-0.736	**0.005**	N.A.	A228001-101	N.A.	N.A.	N.A.	N.A.
-0.2569	n.s.	-0.570	**0.005**	-0.764	**0.004**	N.A.	A231002-101	N.A.	N.A.	N.A.	N.A.
-0.2273	n.s.	-0.562	**0.005**	-0.750	**0.006**	N.A.	A250001-101	N.A.	N.A.	N.A.	N.A.
-0.4355	*0.077*	-0.542	**0.014**	-0.842	**0.000**	N.A.	A251003-101	N.A.	N.A.	N.A.	N.A.
-0.3082	n.s.	-0.561	**0.008**	-0.567	**0.039**	N.A.	A300001-101	N.A.	N.A.	N.A.	N.A.
-0.0566	n.s.	-0.630	**0.002**	-0.796	**0.003**	N.A.	A311002-101	N.A.	N.A.	N.A.	N.A.



Supplemental Figure 1. Hierarchical clustering of changes in metabolite pool sizes in the five parental accessions during 14 days of cold acclimation at 4°C. Normalized peak areas were scaled to unit variance and Euclidian distance was used for clustering. Red indicates the smallest and yellow the biggest differences in metabolite content between samples from nonacclimated and cold acclimated plants.

5. General discussion

5.1. Heterosis in the freezing tolerance of *A. thaliana* hybrids

Plants differ in their ability to withstand low temperatures and freezing according to the climate in their original habitat. While for example *Arabidopsis* or spinach survive sub-zero temperatures, tomato or cucumber are severely harmed by chilling already (Xin and Browse, 2000). Freezing tolerance is a multigenic quantitative trait. *A. thaliana* is distributed over the whole northern hemisphere and it was shown previously that accessions from various origins exhibit different freezing tolerance (Tab. 3). This was tested for non acclimated (NA) and acclimated (ACC) plants after 14 days at 4°C in a cold phytotron (Hannah *et al*., 2006; Rohde *et al.,* 2004).

Table 3: Lethal temperature (LT50) for non acclimated (NA) and acclimated (ACC) plants of eight accessions used to perform reciprocal crosses. LT_{50} was calculated based on electrolyte leakage measurements with detached leaves.

Ecotype	Origin	LT_{50} NA (°C)	LT_{50} ACC (°C)
C24	-----	-4.6	-6.4
Can	Canary Islands	-4.6	-6.8
Co-2	Coimbra, Portugal	-4.8	-6.4
Cvi	Cape verde Islands	-5.4	-6.4
L*er*	Germany	-5.1	-7.0
Col-0	-----	-5.8	-9.3
Rsch	Russia	-6.2	-10.4
Te	Finland	-7.2	-12.2

The temperature range of freezing tolerance in plants is genetically fixed, but in some plant species it is in addition modified by environmental conditions. These plants are able to increase their freezing tolerance during exposure to low, but above-zero temperatures for a certain time. This process is termed cold acclimation and it concerns mostly plants from temperate or cold regions.

In my project, reciprocal crosses were manually performed between C24, Columbia-0 and five other natural accessions (Tab. 3). Freezing tolerance was tested by measuring the lethal temperature (LT_{50}) where 50% of leaf cells were irreparably damaged by freezing before and after a cold acclimation period of 14 days, where the plants were exposed to a constant

temperature of 4°C. Significant differences were found in leaf freezing tolerance between both conditions, between parental lines and F1 hybrids and also between several hybrids.

The results were used to calculate Mid-Parent-Heterosis (MPH) and Best-Parent-Heterosis (BPH). Heterosis occurs in F1-hybrids, but decreases in the following generations with increasing homozygosity due to inbreeding. In allogamous plants, with a wider gene pool, the effect emerges stronger then in selfing plants like *A.thaliana*. Nevertheless, heterosis was found in *Arabidopsis* before, for other traits than freezing tolerance, such as phosphate uptake efficiency, biomass or rosette diameter (Narang & Altmann, 2001; Barth *et al.*, 2003; Meyer *et al*., 2004).

In the present study both kinds of heterosis were found for freezing tolerance, with MPH more frequent than BPH. This confirms former findings of hybrid vigor in freezing response (Rohde *et al*., 2004) of reciprocal C24 x Col-0/ Col-0 x C24 – crosses. I found heterosis values comparable to the former study, stronger in acclimated than nonacclimated plants (Tab. 4) and decreasing in the F2 as it is known from heterosis in several other traits.

Loss of heterosis in subsequent generations could be linked to inbreeding depression, which often appears in hybrids of homozygous parental lines. Nevertheless heterotic effects were not always significant even in the F1. Crosses with strong variation in parental freezing sensitivity never outperformed the better parent (Rsch, Te), while crosses from two intermediate accessions (C24 x Col, C24 x Co-2) exhibited BPH. Possibly there exists a perfect parental genetic distance which increases the probability of strong heterosis in the F1. This might be different for varying traits. Meyer *et al.* (2004) found increasing biomass heterosis in Arabidopsis weakly correlated with an increase in genetic distance, contrary to my results. I found a weak negative correlation between heterosis in freezing tolerance and parentalgenetic distance. An alternative hypothesis could assume a maximum in achievable freezing tolerance for each plant species, reached by Tenela and Rschew and therefore not enhanceable.

Freezing experiments were repeated three times distributed over a year and also seeds freshly produced by repeated manual pollination. Despite changes in LT_{50} (NA, ACC) between replicated experiments, the strength of heterosis was unchanged. Acclimation capacity (i.e. the difference in freezing tolerance between nonacclimated and acclimated plants) also stayed unchanged between the experiments. This might be due to the fixed light and temperature conditions in the cold phytotron over the year. Changes in freezing tolerance can also be ascribed to the varying temperatures during sample preparation. In a non-acclimated laboratory a summer-winter temperature gradient could lead in summer to a faster de-acclimation of the plants or a difference between reciprocal accessions. Wanner and Juntilla (1999) showed a total loss of the freezing tolerance gained during acclimation after only 1-2 days.

Table 4: Mid-Parent-Heterosis (M, blue) and Best-Parent-Heterosis (B, orange) in freezing tolerance (LT_{50}), sugar and proline content for 24 reciprocal crosses under both conditions (na, acc).

Eco-type	LT_{50} na	Glc na	Frc na	Suc na	Raf na	Pro na	LT_{50} acc	Glc acc	Frc acc	Suc acc	Raf acc	Pro acc
ColxCan	M	B	M	-	B	-	-	-	-	-	-	-
CanxCol	M	-	-	M	-	-	-	-	-	-	-	-
C24xCan	-	B	B	B	M	-	B	B	B	B	-	-
CanxC24	-	B	B	B	M	-	B	B	B	B	B	-
ColxCo	M	-	-	M	-	-	B	M	M	-	M	B
CoxCol	-	M	-	-	M	-	-	B	B	B	M	M
C24xCo	B	M	B	-	B	B	B	B	B	B	B	-
CoxC24	B	-	M	-	B	B	B	B	B	B	B	M
ColxCvi	-	M	-	-	-	M	-	-	-	-	M	-
CvixCol	-	M	-	-	-	-	-	-	-	-	M	B
C24xCvi	M	-	-	B	M	-	B	B	B	B	M	M -
CvixC24	M	B	B	B	B	-	B	B	B	B	B	B
ColxLer	-	M	M	M	M	M -	-	-	-	-	-	-
LerxCol	-	-	-	-	-	-	-	-	-	-	-	-
C24xLer	M	-	-	M	M	B	B	M	M	M	M	B
LerxC24	M	M	-	-	M	B	B	M	M	B	M	B
ColxRsch	M	-	-	-	-	M	-	M	M	M	M	M -
RschxCol	-	-	-	-	-	-	-	B	B	B	M	M -
C24xRsch	M	M	M	B	-	-	M	B	B	M	M	M
RschxC24	M	B	M	-	M	-	M	B	B	-	-	M
ColxTe	-	M	-	M	M	M -	-	-	M	-	M	M -
TexCol	-	M	M	-	M	-	-	M	M	M	-	M -
C24xTe	M	B	M	B	B	-	-	M	B	M	M	M -
TexC24	M	B	B	M	B	-	-	B	B	M	B	-

Freezing tolerance (NA, ACC) is affected by external factors like temperature and both light intensity and duration, while hybrid vigor seems to be predominantly genetically determined. This is in agreement with the fact that C24-crosses showed significantly stronger heterosis than Col-crosses. However, strength and occurrence of hybrid vigor increased during cold acclimation. The presumably small optimal genetic distance between parental lines for heterosis in freezing tolerance disagrees with the theory that allogamous plants should show higher heterosis.

5.2. Heterosis in the accumulation of compatible solutes and secondary metabolites

5.2.1. Compatible solutes

Compatible solutes are known to support plant survival under low temperature and other stresses (Jaglo *et al.*, 2001; Klotke *et al.*, 2004; Rohde *et al.*, 2004; Hincha, *et al.*, 2005). The group includes i.e. some amino acids, sugars, sugar alcohols – in general protective compounds that are harmless to the plant independent of the concentration. In this study primary metabolites in F1 and F2 populations (NA, ACC) were determined by HPLC (High performance liquid chromatography) and GC-MS (Gas chromatography-Mass Spectrometry).

Glucose, fructose, sucrose and raffinose were quantitatively measured by HPLC. In agreement with former studies an accumulation during cold acclimation of all four sugars was detected. Sugar content differed according to the treatment (NA, ACC) and the parental accessions. Heterosis in sugar content was stronger in C24-crosses than in Col-crosses and in all crosses heterosis was stronger after cold acclimation than before. Correlations were found between the LT_{50} values of the different F1 hybrids and the content of the single sugars, but also the sum of all sugars. Only of some sugars the separate impact on plant protection was shown yet. For sucrose an enhancement of LT_{50} was shown, tested in plants grown in sucrose enriched nutrient solution (Uemura *et al.*, 2003; Tabaei-Aghdaei *et al.*, 2003). Zuther *et al.* (2004), on the other hand, found no influence of raffinose on freezing tolerance comparing knock out and wild type plants. Probably the protective effect of sugars during freezing arises from the combination of several sugars rather than the specific effect of one sugar.

Proline content also increased after 14 days at 4°C. The level varied strongly between replicates, but clear differences were also found between the accessions and between different F1 hybrids. The increase in proline level in plants in response to cold was repeatedly described (Koster and Lynch, 1992; Xin and Browse, 1998; Xin and Browse, 2001).

Proline either acts as a signal compound which `informs´ the plant about continuing stress or it has its role in secondary responses to cold. It functions after the primary stress and might be a consequence instead of a reason of enhanced freezing tolerance (Wanner and Juntilla, 1999). While sugars start accumulating right after transfer to low temperatures, proline concentration detectably increases first after about 24 hours. In contrast to the sugars, the level of proline stays elevated after return to normal conditions and proline levels are not tightly correlated with freezing tolerance.

Hybrid vigor was calculated not only for freezing tolerance, but also for solute content and significant MPH and BPH found in many hybrids under either both NA and ACC conditions, or only one condition (Tab. 4). Compatible solute heterosis was stronger for C24

than Col-0 crosses and higher in acclimated than in nonacclimated plants. The heterosis effect decreased in the following generation (F2). While proline content and LT_{50} correlated weakly, but significantly, there was no correlation between heterosis in freezing tolerance and in proline quantity. Sugars correlated in content and heterosis with LT_{50} and heterosis in freezing tolerance (Korn *et al.*, 2008).

GC-MS analysis likewise identified increasing proline, glucose, fructose, sucrose and raffinose contents in all accessions and hybrids during cold acclimation. By independent component analysis (ICA) samples from nonacclimated and acclimated plants could be clearly separated into two distinct groups by their metabolite composition (Fig. 2). This agrees with former studies (Hannah *et al.,* 2006; Guy *et al*., 2008). It illustrates the broad impact of cold acclimation on metabolite levels and gives a general impression of the huge network of reactions on transcript and protein level beforehand.

In contrast to the HPLC measurements an absolute quantification is not possible by GC-MS, but relative peak areas can be obtained for all analytes. The content of all four sugar correlated under both growth conditions significantly with freezing tolerance. Proline did not display a significant correlation. A correlation between heterosis in sugar and proline content with LT_{50} heterosis was also not found. Interestingly, most of the 40 metabolites displayed similar correlation patterns. While a majority of the known (METBs) and unknown (MSTs) substances showed a correlation of their pool size with freezing tolerance, a significant correlation between hybrid vigor of both was only found in a few cases.

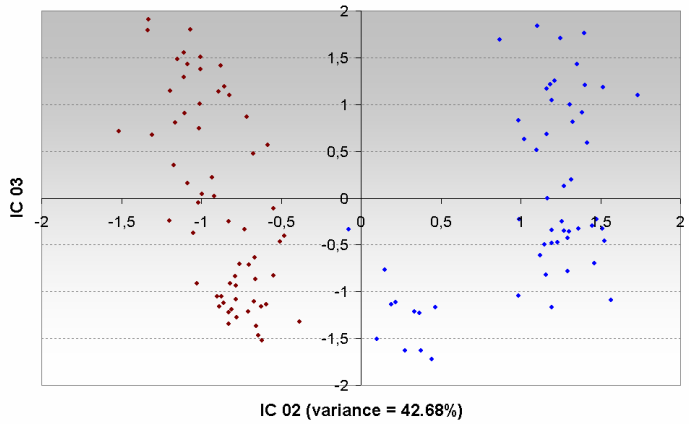


Figure 2: **Independent component analysis (ICA) of non acclimated and acclimated accessions** results obtained by GC-MS analysis from eight F1-crosses and five parental lines, showing a clear separation between samples from nonacclimated (red) and acclimated (blue) plants.

5.2.2. Flavonoids

Secondary metabolites are known to play a role in plant stress tolerance. Flavonoids are pigments that have been shown to protect plants against oxidative, UV or high light stress (Winkel-Shirley, 2002). The accumulation of these pigments is visible by coloring of different plant tissues or organs.

Hannah *et al.* (2006) found the PAP1/ PAP2- regulatory pathway strongly affected under cold. Both PAP1 and PAP2 are transcription factors regulating the expression of genes that encode enzymes involved in flavonoid biosynthesis. The expression of these genes under cold acclimating conditions is correlated with the freezing tolerance of different Arabidopsis accessions (Hannah *et al*., 2006). This finding raised the question of the role of the final products of this pathway, the flavonoids, in freezing tolerance and cold acclimation.

In eight investigated crosses and the related five parental lines, 10 flavonoids could be identified by LC-MS. One of these was found only in Tenela after cold acclimation and was left out in the further analysis. Two of the remaining peaks contain flavonols with quercetin as the aglycon and two contain kaempferol. This was the first analysis of flavonoids in different natural accessions and hybrids of *A. thaliana* and the first analysis of the influence of cold acclimation on this class of secondary metabolites.

We found significant differences between the accessions and the flavonoids under both conditions. The flavonoid content correlated highly significant ($r > 0.8$) with freezing tolerance (NA and ACC). Interestingly the correlation of both kaempferols differed strongly. The role of flavonoids in freezing tolerance is not clear yet. An accumulation is visible through coloring of the leaves of some *Arabidopsis* accessions during cold acclimation. This could be a reaction to the combination of light and cold during acclimation. But this light does not contain UV-B, against which flavonoids protect plants. Flavonols are also known to be effective antioxidants and could thus limit the damage by reactive oxygen species (ROS). Or they are direct players in freezing tolerance inhibiting extracellular ice crystal formation (Scheidt *et al*., 2004).

5.3. The role of the TCA- cycle under cold stress and in heterosis in freezing tolerance

The tri-carboxylic acid (TCA) cycle is central for metabolism and the involved substrates are crucial for animal and plant survival. It acts in oxidative degradation of organic compounds and provides intermediates for other pathways. Most of the involved enzymes and metabolites are the same in aerobic cells of all eucaryotic and procaryotic organisms.

Several studies showed an involvement of the TCA-cycle in various stresses which finally resulted in oxidative stress, like drought stress (Vazquez-Robinet *et al*., 2008), aluminum (Hamel and Appanna, 2001; Mailloux *et al*., 2006), nitrogen (Lancien *et al*., 1999) or manganese (Morgan *et al.*, 2008) stress and of course direct oxidative stress (Janero and Hreniuk, 1996) itself.

In this study a correlation analysis based on the results of global metabolite profiling by GC-MS was performed. Metabolite content was correlated with freezing tolerance and heterosis in metabolite content with heterosis in freezing tolerance. Both calculations were performed for combined data from nonacclimated and acclimated plants.

For most of the detected metabolites a significant correlation between pool sizes quantity in the different genotypes and LT_{50} was shown. In contrast, for only 11 metabolites heterosis correlated with heterosis in freezing tolerance, with $p < 0.1$ and only seven were significantly correlating with $p < 0.05$. Four members of the last group (cis-aconitate, aspartate, fumarate, malate) are part of the TCA-cycle. Isocitrate and succinate belong to the group with lower significance ($p < 0.1$).

This finding is surprising and very interesting. It is the first time heterotic effects can be related to a whole pathway. R-values indicate negative correlations for all six acids, which could mean either a decrease in heterosis of TCA metabolites with increasing heterosis in freezing tolerance or an increase of metabolite heterosis while freezing tolerance heterosis is declining. An improvement in freezing tolerance of F1-hybrids compared to the parental lines would lead to less significant heterotic effects in TCA metabolite accumulation and thereby to a decrease in oxidation. It should be detected if flux into the TCA cycle is reduced or if flux from TCA-cycle is increased.

5.4. Conclusions and Outlook

In this thesis appearance and strength of heterosis in freezing tolerance and corresponding metabolites were investigated in natural accessions and reciprocal hybrids of *A. thaliana*. I started with a comprehensive determination of freezing tolerance and of the accumulation of glucose, fructose, sucrose, raffinose and proline in nonacclimated and acclimated plants.

A significant increase in the content of the compatible solutes was found in all crosses during cold acclimation. Heterosis was stronger in C24- crosses than in Col-crosses, as well as after cold acclimation (ACC) than before (NA). After obtaining this first broad overview, eight crosses were chosen for further metabolite analysis.

I found a clear separation between samples from nonacclimated and acclimated plants in the GC-MS analysis and a highly significant correlation between freezing tolerance and metabolite content for nearly all 40 detected metabolites.

The evaluation of correlations between heterosis in metabolite content and in freezing tolerance indicated a significant role of six compounds of the tri-carboxylic acid (TCA) cycle. This is the first time a whole pathway was shown to be involved in heterotic performance concerning a complex trait.

For the continuation of this study several experiments would be interesting. A transcript profiling of the investigated crosses and accessions under both conditions (NA, ACC) could provide further insight into the process of cold acclimation and heterosis in cold acclimation. This was planned to be integrate into this project but could not be finished because of technical problems. A hybridization scheme was already established (Fig. 3).

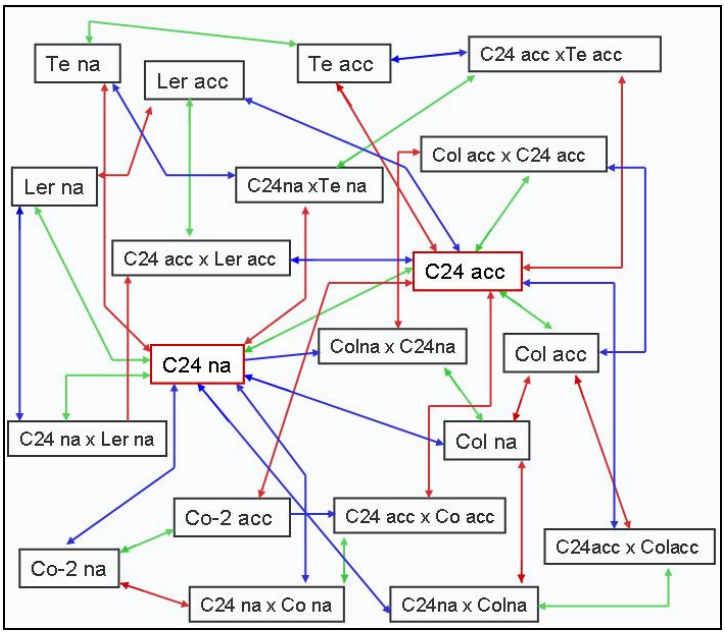


Figure 3: Hybridization design established for transcription profiling with two-colour microarrays. Each color (Red, green, blue) depicts one experiments, each arrow one slide.

The search for heterotic QTLs (hQTLs) could also lead to interesting new findings concerning the molecular basis of heterosis. This is of special interest with regard to the role of the TCA cycle. In addition, radioactively labeled precursors of the TCA cycle could be fed to plants to determine if flux into or from the cycle is accelerated.

To investigate the role of the identified flavonols in freezing tolerance and cold acclimation in more detail, knockout lines or overexpressors with a changed expression of the appropriate genes encoding the PAP1/PAP2 transcription factors or different enzymes of the biosynthetic pathway could be tested with the same experimental setup used in this study.

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7. Deutsche Zusammenfassung

Arabidopsis thaliana ist weltweit eine der meistgenutzten Modellpflanzen. Sie besitzt ein kleines Genom (125 Mb auf 5 Chromosomen), stellt geringe Anforderungen an die Anzucht, hat eine kurze Generationszeit und ist weit verbreitet in der gesamten nördlichen Hemisphäre. Zudem ist sie selbstend und somit ein gutes Objekt für Kreuzungsversuche.

In der vorliegenden Arbeit wurde Heterosis in der Frosttoleranz im Vergleich von *Arabidopsis*- Kreuzungen untersucht. Der Begriff Heterosis beschreibt die Verbesserung von F1- Hybriden gegenüber den Eltern. Es wird zwischen Mid – Parent - Heterosis (MPH), der Verbesserung gegenüber dem elterlichen Mittelwert und Best – Parent - Heterosis (BPH), der Steigerung einer Eigenschaft über den besseren Elternteil hinaus, unterschieden. Dabei geht es beispielsweise um die Erhöhung der Biomasse oder des Samenertrages, aber auch um die Toleranz gegenüber verschiedenen Arten von Stress. Die Verbesserung nimmt in den folgenden Generationen bei Inzuchtlinien wieder ab.

Trotz jahrzehntelanger Nutzung des Prinzips in der landwirtschaftlichen Züchtung ist die genetische, dem Effekt zugrunde liegende Ursache noch immer unklar. Verschiedene Studien deuten auf Dominanz, Überdominanz oder Epistasie hin, allein oder in Kombination.

Die elterlichen natürlichen Akzessionen für meine Heterosisuntersuchungen stammen aus klimatisch sehr unterschiedlichen Regionen. Ihre Frosttoleranz (Lethaltemperatur, LT_{50}) wurde in vorherigen Arbeiten untersucht und eine klare Korrelation mit der Temperatur der ursprünglichen Herkunftsgebiete nachgewiesen. Aus sechs Akzessionen (Canary Island, *Can*; Cape Verde Islands, *Cvi*; Coimbra, *Co-2*; Landsberg *erecta*, *Ler*; Rschew, *Rsch* und Tenela, *Te*) und den Laborlinien C24 und Columbia (*Col-0*) wurden 24 reziproke Kreuzungen erzeugt um Frosttoleranz, Prolin- und Zuckergehalte (Glukose, Fruktose, Saccharose und Raffinose) zu bestimmen. Diese Metabolite akkumulieren nachweislich unter Kälteeinwirkung in Pflanzen. Alle Messungen wurden an nicht akklimatisierten, unter Standardbedingungen angezogenen (NA), und kälteakklimatisierten, für zwei weitere Wochen zu 4°C transferierten (AKK) Pflanzen durchgeführt. Daraus wurden Art und Stärke der Heterosis berechnet und anhand dessen acht Kreuzungen für weitergehende Versuche ausgewählt (C24 x Col, Col x C24, C24 x Co-2, C24 x L*er*, C24 x Te, Col x Co-2, Col x L*er*, Col x Te). Mit diesen und den Eltern wurde (NA und AKK) ein Metabolitprofiling (Gaschromatographie-Massenspektrometrie, GC-MS) durchgeführt und Flavonoidgehalte (Flüssigchromatographie-Massenspektrometrie, LC-MS) analysiert.

Die Untersuchungen zeigten signifikant häufigere und stärkere Heterosis in C24- als in *Col*- Kreuzungen. Das galt sowohl für die ermittelte Frosttoleranz, als auch für die

Akkumulation von Prolin und den vier Zuckern. Die Heterosiswerte in akklimatisierten Pflanzen überstiegen die nicht akklimatisierter Pflanzen um ein Vielfaches. Gleiches wurde für die Flavonoidgehalte gefunden.

Korrelationsanalysen zeigten deutliche Korrelationen zwischen der Frosttoleranz und den Gehalten der Kohlenhydrate, Aminosäure und mehrerer Flavonole. Signifikant korrelierten auch die Stärke der Heterosis in der Frosttoleranz und in den Metabolitgehalten.

Das globale Metabolitprofiling via GC-MS bestätigte die oben genannte Akkumulation und die zuvor errechneten Korrelationen für die vier Zucker und Prolin. In drei unabhängigen Experimenten wurden übereinstimmend 40 Metabolite detektiert und untersucht. Die stärkste Akkumulation während der Kälteakklimatisierung zeigten C24 und Coimbra, die geringste Tenela. Viele der Substanzen korrelierten signifikant mit der Frosttoleranz und/oder die MPH in den Metabolitgehalten mit der MPH in der LT_{50}. Interessanterweise waren sechs von 11 der signifikant korrelierenden Substanzen wichtige Komponenten des Citratzyklus. Eine Veränderung der Menge verschiedener Teile dieses Stoffwechselweges wurde bereits in anderen Organismen (Pflanzen und Tieren) und im Zusammenhang mit anderen Streßarten beobachtet, jedoch nicht im Zusammenhang mit Heterosis. Die hier vorliegenden Ergebnisse zeigen eine negative Korrelation zwischen MPH in Frosttoleranz und Metabolitakkumulation. Das deutet auf eine verstärkte Rolle von Teilen des Citratzyklus in den Kreuzungen im Vergleich zu den Eltern hin und somit auf eine Veränderung der Flußraten im Zyklus.

Weiterführend wäre es interessant ein Transkriptprofiling durchzuführen, das die Genexpression der Kreuzungen und Eltern bevor und nach der Kälteakklimatisierung vergleicht. Eine ähnliche Untersuchung gibt es bereits als Vergleich mehrerer natürlicher homozygoter Linien von Hannah *et al.* (2005). Des Weiteren könnte eine Analyse quantitativer Eigenschaftsloci (QTLs) Aufschluß darüber geben, wie der Citratzyklus metabolisch und genetisch mit der gefundenen Heterosis in Verbindung steht.

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VI List of publications

Marina Korn, Silke Peterek, Hans-Peter Mock, Arnd G. Heyer & Dirk K. Hincha (2008) "Heterosis in the freezing tolerance, and sugar and flavonoid contents of crosses between *Arabidopsis thaliana* accessions of widely varying freezing tolerance" Plant, Cell and Environment **31**, 813-827

Marina Korn, Tanja Gärtner, Alexander Erban, Joachim Kopka, Joachim Selbig, Dirk K. Hincha "The metabolic basis of *Arabidopsis thaliana* freezing tolerance"

Submitted Manuscript at Nature Genetics, July 2008

VII Conferences

Conferences

Talks

Marina Korn, Arnd G. Heyer, Dirk K. Hincha *"*Heterosis in the frost tolerance of *Arabidopsis thaliana* accessions*", SEB Meeting 2007, Glasgow/ Great Britain*

Marina Korn, Arnd G. Heyer, Dirk K. Hincha "Heterosis in the frost tolerance of *A.thaliana*- crosses", *XVII. Berliner Botanischen Graduierten-Kolloquium "Havel-Spree-Kolloquium" 12.2006, Potsdam/ Germany*

Posters

Marina Korn, Arnd G. Heyer, Dirk K. Hincha *"*Heterosis in the frost tolerance of crosses between different *Arabidopsis thaliana* accessions*" SEB Meeting 2007, Glasgow/ Great Britain*

Marina Korn, Arnd G. Heyer Dirk K. Hincha "Heterosis in the freezing tolerance of crosses between different accessions of *Arabidopsis thaliana", Keystone Symposium 2006, Colorado/ USA*

Workshops

05. – 07.09.2007 **"**Workshop for Molecular Interactions"
Methods and Technologies of Genomics
Free University of Berlin, Berlin

25. - 26.10.2006 „Symposium on Plant Evolution and Domestication"
Max-Planck-Institute for Plant Breeding, Cologne

VIII Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig am Max-Plack-Institut für Molekulare Pflanzenphysiologie angefertigt habe und keine weiteren als die angegebenen Hilfsmittel und Quellen genutzt habe.
Ich versichere, dass die vorliegende Doktorarbeit an keiner anderen Hochschule als der Humboldt Universität zu Berlin eingereicht wurde.

Marina Korn

Berlin, 07.07.2008